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In vivo and in vitro degradation of tissue engineered collagen and mineralised collagen scaffolds used in bone tissue engineering

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IN VIVO AND IN VITRO DEGRADATION OF TISSUE ENGINEERED COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS USED IN BONE TISSUE ENGINEERING

Dr. Stephen M Kieran MB BCh BAO, MRCSI, DOHNS

A dissertation submitted to the Royal College of Surgeons in Ireland (NUI) for the degree of

MCh

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THESIS DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher MCh degree is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research program this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Stephen Kieran.

Student Number 08512671

Date 17th March, 2010

ABSTRACT

A clinical need exists to provide alternatives to autologous bone grafting for the reconstruction of head and neck bone defects. This thesis examines the utility and success of a tissue engineering approach to this problem using novel collagen based scaffolds combined with mesenchymal stem cells.

The objective of this work was to determine how the addition of a calcium phosphate mineral phase to a collagen-based scaffold, designed for use in bone tissue engineering, affects the in-vitro and in-vivo scaffold degradation characteristics in addition to the rate of tissue healing as assessed by new bone formation and host immune response in a rat calvarial model.

In vitro analysis of the collagen calcium phosphate (CCP) scaffolds revealed minimal degradation and loss of mechanical properties over time in a non enzymatic degradation media, these results were similar to those of pure collagen (Collagen) and collagen glycosaminogylcan (CollGAG) scaffolds. However, in a bacterial collagenases media the mineralised CCP scaffolds were relatively resistant to degradation compared to the collagen and CollGAG scaffolds.

The CollGAG and CCP scaffolds were then implanted into a 7mm trans-osseous critically sized defect created in the calvarium of Wistar rats. Half of each group were pre-cultured with mesenchymal stem cells (MSC). Animals were sacrificed at 4 and 8 weeks post implantation. Quantitative histomorphometry identified significantly better rates of new bone formation in non MSC seeded scaffolds, with superior results for the mineralised collagen scaffold at 8 weeks (37.24%V13.15%, $p<0.05$). Scaffolds pre cultured with MSCs showed an accumulation of fibrous tissue at the periphery of the scaffold.

In the knowledge that macrophages play an important role in fracture healing and that this fibrous tissue surrounding the MSC seeded scaffolds appeared

inflammatory in nature, immunohistochemical staining was performed to confirm the presence of macrophages (CD68) and to phenotype the macrophage response (CD163, CCR7). A marked macrophage response to the MSC seeded scaffolds, with only a moderate response to non seeded implants was seen. Whilst all scaffold types demonstrated an M2 (immunomodulatory and tissue remodelling) macrophage phenotype response the location of this response was confined to the scaffold periphery in the MSC seeded group as opposed to areas of new bone formation in the non seeded group.

In conclusion this thesis demonstrates quantitatively superior new bone formation in non MSC seeded mineralised (CCP) collagen scaffolds. Aside from increasing the scaffolds mechanical properties the addition of a mineral phase also retards scaffold degradation. Furthermore, an appropriate macrophage response is necessary for successful bone deposition in collagen scaffolds and appears hindered by current tissue engineering approaches.

TABLE OF CONTENTS

FIGURE LEGEND

STANDARD ABBREVIATIONS

BMP Bone morphogenetic protein

$CaCl_2$ Calcium chloride

CCP Collagen Calcium Phosphate

CD Cluster of differentiation

ColIGAG Collagen Glycosaminoglycan

CCP Collagen Calcium Phosphate

CO^2 Carbon Dioxide

CP Calcium Phosphate

DHT Dehydrothermal

DMEM Dulbecco's modified Eagle's medium

ECM Extracellular Matrix

EDAC 1-ETHYL-3-(3-DIMEHTYLAMINOPROYPL)CARBODIIMIDE

EDTA Ethylenediaminetetraacetic acid

GAG Glycosaminoglycan

HA Hydroxyapatite

H&E Haematoxylin and Eosin

MMP Matrix metalloproteinase

mRNA Messenger RNA

$NaNH_4HPO_4$ Ammonium sodium hydrogen phosphate

PBS Phosphate buffered saline

PCL Polycaprolactone

PGA Polyglycolic acid

PLGA Poly(lactic-co-glycolic acid)

PLLA Polylactic acid

SD Standard Deviation

SIS Small Intestinal Submucosa

TE Tissue Engineering

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I would like to thank a number of people for the help, support and encouragement that I have received in performing this work and preparing this thesis.

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This work could not have been performed without the help of my collaborators Mr. Frank Lyons, Dr. Amir Al-Munajjed and Dr. John Gleeson. Dr. Mary Toner's help in performing the histological assessment is also greatly appreciated. I would like to recognise the invaluable assistance and encouragement from Johnny, Grainne, Sonia, Tanya, Paul, Niamh, Mike, Orlaith and Ciara in the department of anatomy of the RCSI.

CHAPTER 1 – LITERATURE REVIEW

1.1 OVERVIEW

At present, reconstruction of head and neck skeletal defects, such as the mandible, orbital floor or cranium is performed using autologous bone grafts or inert non-degradable biomaterials such as metals. Neither of these solutions is totally satisfactory. Bone grafts are limited by the amount of donor tissue available and donor site morbidity. Non degradable biomaterials are associated with poor long term host integration and potential extrusion. Hence, one research focus has turned to development of bone by tissue engineering techniques.

Modern tissue engineering aims to restore tissue function by combining cells on 3D scaffold materials under the influence of signalling molecules which encourage and facilitate cell growth. Such a living tissue construct aims to be functionally, structurally and mechanically equal to the tissue it has been designed to replace. The scaffold acts as a template for tissue formation and must fulfil a series of specific criteria. In general the scaffold should be highly porous, with a high surface area and have a specific 3D shape to fit the clinical defect. These characteristics have been found to permit optimum cell attachment, cell migration, cell proliferation and cell differentiation. The rate at which a scaffold degrades can also influence cell growth, tissue regeneration, and host cellular responses. Ideally the scaffold should degrade at a rate similar to the rate of new tissue formation. Collagen scaffolds degrade by both hydrolysis and enzymatic digestion and degradation is

slower in scaffolds that have been cross linked or have a GAG component (Weadock, Miller et al. 1996; Pek, Spector et al. 2004). If degradation is too fast, the structural support for the proliferating cells will be lost and if degradation is too slow is can impose a physical barrier to new tissue growth.

The optimum scaffold material for new bone formation has yet to be established. Scaffolds manufactured from natural polymers such as collagen are highly porous and readily biodegradable but lack the mechanical properties of bone. In view of these mechanical limitations novel collagen based scaffolds which aim to be both degradable and exhibit improved mechanical properties have been developed in our laboratory by various techniques including; the addition of a cross linking step to bind collagen fibres together, the addition of chondroitin-6-sulphate and the addition of a mineral phase to the scaffold, such as calcium phosphate (O'Brien, Harley et al. 2004; O'Brien, Harley et al. 2005; O'Brien, Harley et al. 2007; Al-Munajjed, Gleeson et al. 2008). Two of these mechanically superior collagen based scaffolds (collagen GAG and collagen Calcium phosphate) are the subject of this thesis.

Mesenchymal stem cells (MSCs) derived from host bone marrow have the ability to differentiate into several cell types including chondrocytes and osteoblasts. Tissue engineered scaffolds seeded with MSCs have shown promise in the field of bone tissue engineering with significant levels of new bone formation (Dyson, Genever et al. 2007; Mandal and Kundu 2009).

Stem cell seeded scaffolds are however, reported to develop a dense inflammatory capsule at their periphery that inhibits neo-vascularisation and nutrient flow (Valentin, Badylak et al. 2006; Badylak, Valentin et al. 2008). Macrophages not only play a role in the host immune response to implanted scaffolds but have an important role in the initial inflammatory phase of fracture healing. Cognisant that macrophage recruitment is an important phase in fracture healing and that scaffold materials have been associated with an inflammatory response, this study undertook to determine the macrophage response to mechanically optimised collagen based scaffolds with and without pre-culture with mesenchymal stem cells.

In summary, this thesis examines the in vitro and in vivo characteristics of two novel collagen based scaffolds. The rate of new bone formation and scaffold degradation after implantation in an animal model is quantitatively assessed for scaffolds with and without the addition of mesenchymal stems cells. Furthermore, the role played by macrophages in successful new bone formation is demonstrated.

1.2 BIOMATERIALS & TISSUE ENGINEERING IN OTOLARYNGOLOGY

A significant proportion of modern surgical practice aims to restore function by replacing damaged or diseased tissues and organs, either by using artificial implants or by transplantation of tissues.(Vacanti and Langer 1999). Otolaryngology as a surgical speciality has been at the forefront of the adoption of such techniques with autotransplantation widely used in otologic and rhinologic surgery (i.e. temporalis fascia graft, fascia lata graft, small bowel free flap, conchal cartilage graft) however such procedures may be hindered by factors such as immune rejection, limited graft tissue supply and donor site morbidity.

The field of regenerative medicine which encompasses tissue engineering aims to regenerate tissues and restore organ function by implantation of cells and/or tissue grown outside the body or stimulating cells to grow into an implanted matrix. Tissue engineering has developed from the use of biomaterials to repair or replace diseased tissue, to the modern practice of using controlled 3D scaffolds in which cells are seeded under the control of signalling molecules; the so called tissue engineering triad (**Figure 1.1**) (Stock and Vacanti 2001). Such a cell seeded scaffold is henceforward termed a construct.

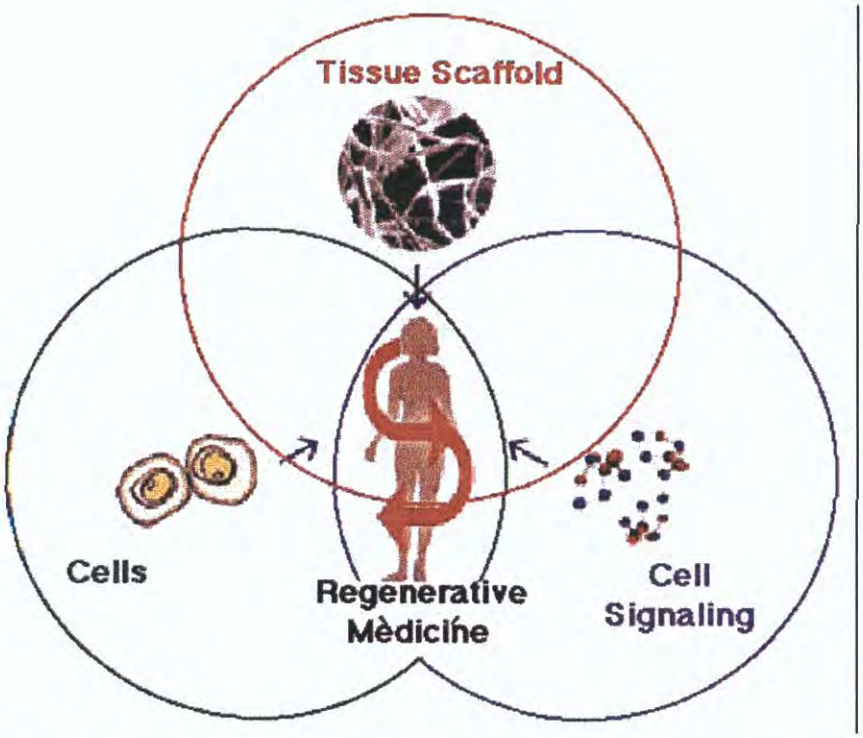

Figure 1.1 The tissue engineering triad indicating the key factors in the tissue engineering process. (bme.biomed.dal.ca).

In Otolaryngology, particularly the sub-speciality of otologic surgery, significant experience has been developed in the use of non tissue engineered biomaterials, especially for middle ear reconstruction. Initial attempts at ossicular reconstruction, using biomaterials, were marred by graft extrusion (Portmann 1967). In the 1980's the development of hydroxyapatite ossicular prosthesis heralded an era of technically successful middle ear reconstructions (Wehrs 1995). Hydroxyapatite and metals such as titanium subsequently developed applications in head and neck skeletal reconstruction, particularly cranial defects, orbital floor fractures and mandible reconstruction. However, as these biomaterials are not biodegradable, they are associated with concerns about long term implant failure due to mechanical mismatch at the implant tissue interface. Ideally an implant

material should be biodegradable, ultimately being replaced by regenerated tissue, thus obviating these concerns.

Aside from biodegradability, the advantage of a tissue engineered construct is that tissues can be designed to grow in such a way that they precisely match the requirements of the individual in terms of size, shape and immunologic compatibility, minimizing the need for further treatment (Stock and Vacanti 2001). Examples of tissue engineered constructs of relevance to the Otolaryngologist, currently under development include bone (with which this work is primarily concerned), cartilage for rhinoplasty and airway reconstruction (Macchiarini, Jungebluth et al. 2008; Weidenbecher, Tucker et al. 2008), tympanic membrane for repair of tympanic membrane perforations (Parekh, Mantle et al. 2009), nerve for reconstruction of the facial nerve following trauma, iatrogenic damage or sacrifice (Mosahebi, Fuller et al. 2002; Guo and Dong 2009) and vocal cord repair (Gilbert, Agrawal et al. 2009) (**Figure 1.2**)

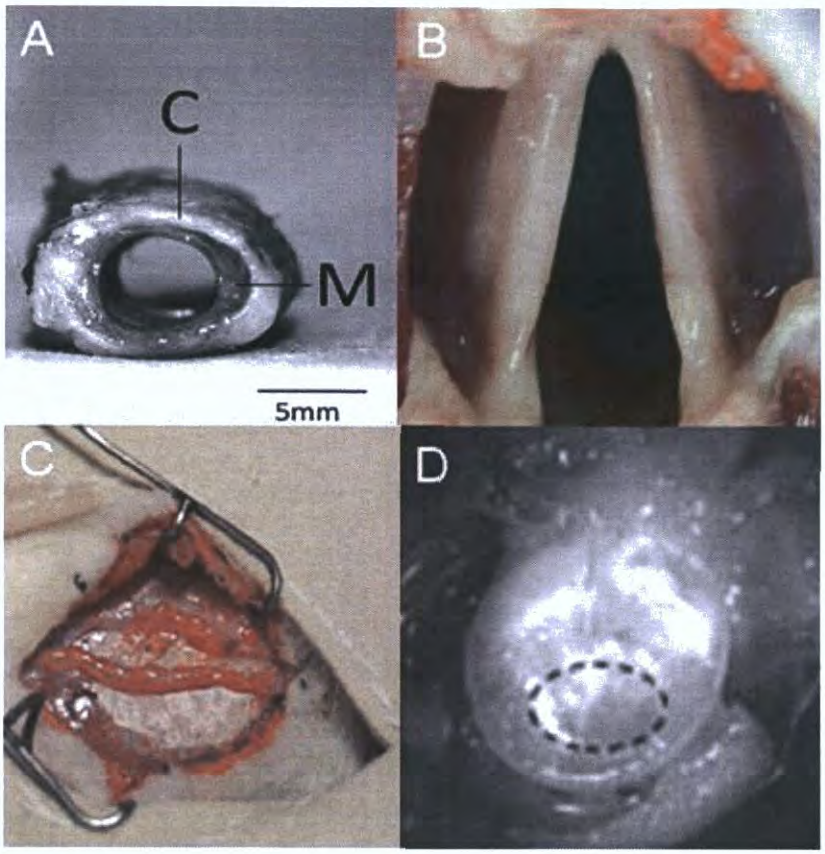

Figure 1.2. *Examples of tissue engineered constructs currently being investigated for clinical applications in otolaryngology. (A) Autologous neotracheal constructs implanted in rabbit abdomens (Weidenbecher, Tucker et al. 2008). (B) Repair of acute vocal cord injuries in a canine model (Gilbert, Agrawal et al. 2009). (C) Repair of a 6mm facial nerve defect (buccal branch) in an animal model (Guo and Dong 2009) (D) Repair of chronic tympanic membrane perforation in a chinchilla model (Parekh, Mantle et al. 2009).*

Some early reports now testify to experimental use of tissue engineered constructs in human patients for indications applicable to the field of otolaryngology. Reconstruction of a human mandible defect using a polycaprolactone (PCL) scaffold, infiltrated with platelet-rich plasma and recombinant human bone morphogenetic protein-2 has been reported in a single case with a history of periimplantitis. However this implant has to be individually fashioned on a case-by-case basis and has poor degradability, requiring approximately six months for scaffold degradation (Schuckert, Jopp

et al. 2009). Whilst Macchiarini et al. reported the implantation of a mesenchymal stem cell seeded cadaveric tracheal segment into a 30 year old female with end stage bronchomalacia (Macchiarini, Jungebluth et al. 2008) (**Figure 1.3**).

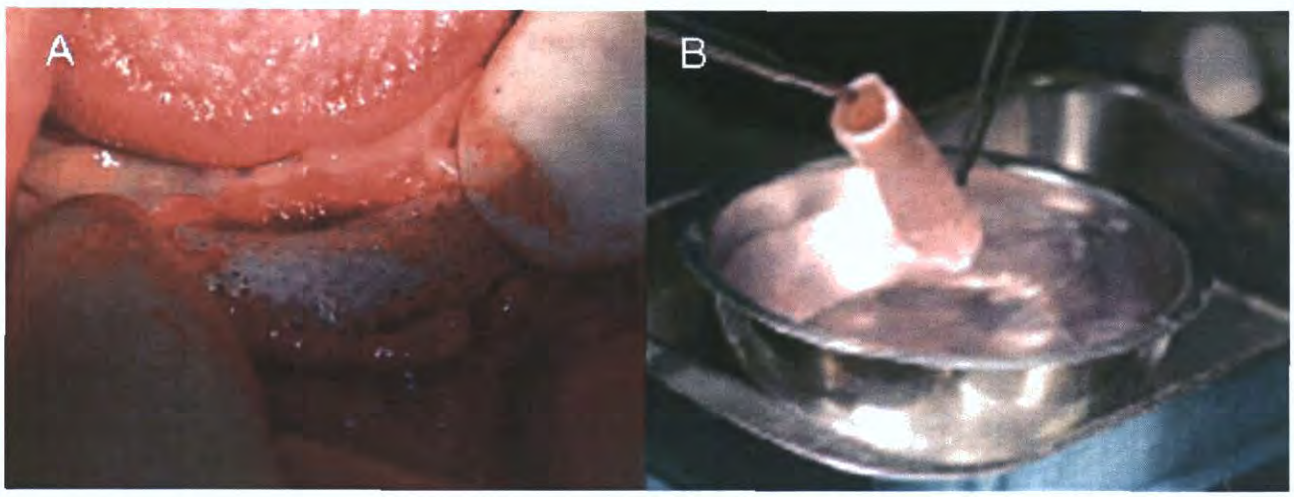

Figure 1.3. *(A) Example of mandible reconstruction using a polycaprolactone scaffold in a human patient (Schuckert, Jopp et al. 2009). (B) Tissue engineered cadaveric tracheal graft prior to insertion into a 30 year old female with bronchomalacia (Macchiarini, Jungebluth et al. 2008).*

1.3 BONE AND BONE GRAFTS

Bone is comprised of a mineral phase, collagen and cells. Two thirds of the mass of bone is a mineral, which is made up of hydroxyapatite (HA, Ca10(PO4)6(OH)2) crystals, and small amounts of carbon, sodium, magnesium and fluoride. Roughly one third of the mass of bone is collagen fibres and cells make up about 2% of the mass. (Martini 2006)

Aside from this intercellular mineralised matrix, bone is composed of four cell types (**Figure 1.4**); osteocytes, which are found in cavities (lacunae) within the matrix; osteoblasts which synthesise the organic components of the matrix; and osteoclasts, which are multinucleated giant cells involved in the resorption and remodelling of bone tissue. Osteoprogenitor cells are immature progenitor cells located in periosteum that produce osteoblasts. Osteoclasts derive from the monocyte / macrophage system (via Mesenchymal Stem Cells). The term "osteogenic cells" encompass all cells that have the ability to produce bone.

Since metabolites are unable to diffuse through the calcified matrix of bone, the exchanges between osteocytes and capillaries depend on communication through the canaliculi, thin cylindrical spaces that perforate the matrix. All bones are lined on both internal and external surfaces by layers of tissue containing osteogenic cells. Endosteum on the inner surface and periosteum on the outer surface. (Junqueira, Carneiro et al. 1995)

Figure 1.4 *Cell types in bone(Martini 2006)*

New bone can be formed in two distinct ways. Intramembraneous ossification is the process of new bone formation via direct mineralisation of matrix secreted by osteoblasts. Whereas, endochondral ossification involves the deposition of bone matrix on a pre-existing cartilage matrix. The product of both processes is primary or immature bone, a temporary tissue that is ultimately replaced by definitive lamellar or secondary bone. Flat bones, such as the frontal and parietal bones of the skull, parts of the occipital and temporal bones, the mandible and maxilla are formed by intramembranous ossification whilst long bones are formed by endochondral ossification. During bone growth, areas of primary bone, areas of resorption, and areas of lamellar bone appear side by side. This combination of synthesis and removal (termed bone remodelling) occurs not only in growing bones but also throughout adult life, although the rate of turnover in adulthood is considerably slower. (Junqueira, Carneiro et al. 1995)

When bone is fractured, bone matrix is destroyed and bone cells adjoining the fracture die. The damaged blood vessels produce a localised haemorrhage with clot formation. During repair the blood clot, cells and damaged bone matrix are phagocytosed by macrophages during the initial inflammatory phase. The periosteum and endosteum around the fracture respond with intense proliferation of osteoprogenitor cells, which form a cellular tissue surrounding the fracture and penetrating between the extremities of the fracture bone. Primary bone is then formed by endochondral ossification of small cartilage fragments that appear in the connective tissue of the fracture. Bone is also formed by means of intramembranous ossification. Therefore,

areas of cartilage, intramembranous ossification and endochondral ossification occur simultaneously in a healing fracture.

Such fracture healing is limited by the size of a fracture. If the gap in the bone is too large, it cannot be bridged and non-union will occur, this size threshold is termed the critical defect size. The animal models designed to assess the utility of new therapies designed to aid fracture healing (such as our collagen based scaffolds) utilise this concept of a critical size defect. If the experimental product results in healing of a critically sized bone defect, it has therefore been shown to be superior to normal physiological fracture healing.

Since the 19th century, techniques for successful auto-grafting of bone have been developed to treat bone defects greater than the critical size where non healing is inevitable or already occurred. Autogenous bone grafting remains the clinical gold standard to the present day. The most common donor sites, for bone grafts being the fibula and iliac crest. Whist fibular grafts have the advantage of potentially being incorporated into an osteocutaneous free flap suitable for reconstruction of large mandible and/or facial defects, widespread bone grafts use is hindered by donor tissue availability and donor site morbidity.

Historical alternatives to the autologous graft are allografts, obtained from a different individual of the same species (organ donors). The drawback to this option is the danger of transmission of viral and potentially prion mediated infection. To overcome this danger, demineralised bone matrix has been used with some success as the allograft remains both osteoinductive and

osteoconductive whilst acellular (Lee, Roper et al. 2005). Xenografts, acquired from other species, are another alternative. However, the morphology of the bone is different between different mammals and the risk of cross-species infection exists. Coral has also been used as an alternative to bone grafts but has a structure very different to that of bone and hence osteointegration of this type of graft has proved difficult (de la Caffiniere, Viehweger et al. 1998).

1.4 TISSUE ENGINEERING OF BONE

In view of the limited supply, the potential for donor site morbidity and the inherent limitations of allografts and non-tissue engineered materials such as coral, the development of techniques to develop tissue engineered bone or novel bone replacement materials have both a clinical necessity and a commercial impetus.

The proposed site of implantation of a tissue engineered construct is important in the development of bone replacement products. Mechanical strength is of critical importance in load bearing areas (long bones) whilst less significant in non-load bearing areas such as the skull. Clinical scenarios where such bone graft alternatives are required in the field of Otolaryngology / Head and Neck surgery are in craniofacial reconstruction, reconstruction of orbital floor fractures (following trauma) and mandible reconstruction post resection of oral cavity malignancies or complicated dental infections. These head and neck regions are all non-weight bearing areas of the skeleton and therefore do not necessitate the mechanical properties of constructs designed

for long bone defects. However, they are subject to strong muscle forces from the muscles of mastication, and have thus traditionally required osseo-integrated implants.

In order for osteoblasts to undergo successful new bone formation, the scaffold material most be manufactured in such a way that it has a similar 3D anatomical shape to the defect being repaired. For some materials this requires specific scaffold machining based on pre-operative CT scanning, whereas softer scaffold materials can be more easily tailored to shape and size at the time of implantation.

A further site specific factor when designing tissue engineering constructs for the head and neck is that they should be easily handled surgically and be compatible with the local environment. For example, a scaffold designed for implantation in the mandible should be at least partially resistant to degradation by saliva as this is an inevitable contaminant of surgical insertion.

1.5 SCAFFOLDS FOR BONE TISSUE ENGINEERING

A high porosity and good interconnectivity that allows cellular infiltration and supports vascularisation is needed in a bone graft substitute. Cells must be able to penetrate into the centre of the graft forming a homogeneously distributed cell-graft construct. A high porosity and interconnectivity also ensures the transport of nutrients to and waste removal from cells (O'Brien, Harley et al. 2004). Additionally, a high surface area improves bone growth by increasing protein adsorption in non-biological materials.(Woodard,

Hilldore et al. 2007) In the absence of bone cells (osteoclasts, osteoblasts and osteocytes) in the graft, remodelling of the bone cannot occur throughout the graft. It may occur by creeping substitution at the interface between the graft and the bone, as bone cells have access to this area. However, this process is slow and remodelling of the entire graft is preferable.

Mechanical properties of scaffolds are not only important to facilitate the handling of the scaffolds and support of local tissues, but also influence cellular activity and proliferation (Yeung, Georges et al. 2005; Engler, Sen et al. 2006). The differentiation of mesenchymal stem cells (MSC) has been shown to be influenced by the mechanical properties (compressive strength) of the scaffold they were seeded on. Neurogenic markers are found on substrates with Young's moduli of 1kPa, myogenic on substrates with Young's moduli of 11kPa and osteogenic markers at Young's moduli of 34 kPa respectively (Engler, Sen et al. 2006) (Young's moduli being a measure of stiffness of an elastic material. A higher Young's modulus corresponds to a stiffer material).

The size of pores in scaffolds is also a main focus of current research due to its influence on cell seeding, attachment, infiltration and growth. Common pore sizes are from 10μm to 800μm(Sachlos and Czernuszka 2003). O'Brien *et al.* have shown that in collagen-glycosaminoglycan scaffolds pores need to be large enough to allow cells to migrate into the structure (about 20μm) but small enough (about 100μm) to establish a sufficiently high specific surface to allow a minimal ligand density required for efficient binding of a critical number of cells to the scaffold (O'Brien, Harley et al. 2005). The optimum pore size

required for new bone formation and vascularisation is cell and tissue dependent (Cao, Mitchell et al. 2006; Byrne, Farrell et al. 2008), with optimum pore size for CGAG scaffolds in bone tissue engineering of either 120 μm or 325 μm shown in in-vitro osteoblast studies (Murphy, Haugh et al.).

As the preferred scaffold characteristics are different with each specific application, many materials have been proposed as scaffolds suitable for tissue engineering. There are three major classifications of materials that have been used as scaffolds for tissue engineering: ceramics, polymers (synthetic and natural) and composite scaffolds which combine both ceramic and polymer components into a single scaffold.

CERAMICS

Ceramics are crystalline in nature and formed from inorganic non-metallic compounds, which are stiff but brittle materials. Because of their high stiffness ceramic scaffolds are commonly used in bone tissue engineering. Calcium phosphate (CP) is the name of a family of minerals containing calcium ions and orthophosphates, meta-phosphates and occasionally hydrogen. Characteristics of ceramics include hardness, high compressive strength and high melting points. Drawbacks of ceramics are that they are brittle, have poor tensile properties and are difficult to fabricate. The most common forms are tri-calcium phosphate (TCP) and hydroxyapatite (HA).(Pilliar, Filiaggi et al. 2001; Porter, Pilliar et al. 2001; Ribeiro, Barrias et al. 2006; Weinand, Pomerantseva et al. 2006) Both have been shown to be highly biocompatible and osteoconductive. They differ however, in their biological response and resorbability. Porous TCP is removed from the implant site as bone grows

whilst HA is less resorbable.(Giannoudis, Dinopoulos et al. 2005) HA scaffolds with a porosity of 0.1-3% were found to be biocompatible when implanted into rat tibiae and the bone bonded strongly to the scaffold. However, after six months, no degradation of the implanted scaffold occurred. Due to the non-porous nature of the implant, cells could not infiltrate into it and no remodelling could occur. (Denissen, de Groot et al. 1980)

POLYMERS

Polymers can be classified as either synthetic or natural. The most commonly used synthetic polymers for scaffold fabrication are polylactide acid (PLLA), polyglycolide acid (PGA) and a combination of the two, poly-lactic-co-glycolic acid (PLGA) which are FDA approved (Lin, Barrows et al. 2003; Taboas, Maddox et al. 2003). The main advantage of using synthetic polymers is the fact that they can be manufactured with a wide tailored range of mechanical properties and architectures. However widespread use of synthetic polymers has been hindered by their degradation products, acidity and alcohol content. In particular, scaffold acidity has been implicated in accelerating the degradation of the scaffold and causing a pronounced inflammatory response in the surrounding tissue leading to an inhibition of tissue formation.

Natural polymers that have been investigated as potential scaffold materials include collagen, chitosan, gelatin, silk fibrin, glycosaminoglycan and elastin (Park, Park et al. 2002; Nazarov, Jin et al. 2004; Dawson, Wahl et al. 2008). In general, as biological materials they have excellent biocompatibility and

non-toxic degradation products. A disadvantage of natural polymers is their poor mechanical properties. One of the most important proteins in connective tissue is collagen. Although more than a dozen types of collagen have been described, the most common and important are types I, II, III, IV and V. collagen type I is the most abundant and occurs in collagen fibres that form bones, dentin, tendons and dermis. Type II collagen is present mainly in hyaline and elastic cartilage, with only very thin fibrils formed. Collagen is a naturally occurring component of articular cartilage and is favoured as a scaffold material for joint tissue repair and replacement (Junqueira, Carneiro et al. 1995).

Type I collagen is often used in tissue engineering (TE) because of its excellent biocompatibility. It can be used alone or combined with glycosaminoglycan, a polysaccharide component of natural bone, in order to improve the structural properties of the scaffold. Collagen glycosaminoglycan (GAG) scaffolds were first developed by Ioannis Yannas at the Massachusetts Institute of Technology for use in skin regeneration (Yannas and Burke 1980). Since then, collagen-GAG (CGAG) scaffolds have shown potential for application in nerve, cartilage and conjunctiva regeneration (Chamberlain, Yannas et al. 1998; Lee, Grodzinsky et al. 2001; Auxenfans, Fradette et al. 2009).

In our laboratory, CGAG scaffolds have been investigated as potential scaffold materials for bone tissue engineering. Farrell *et al.* demonstrated that CGAG scaffolds support the differentiation of MSC down chondrogenic and osteogenic lineages (Farrell, O'Brien et al. 2006). The advantages of these

scaffolds are their high degrees of biocompatibility, porosity (99.5%) and non-toxic degradation products. Furthermore, the natural availability of binding sites upon collagen allows the scaffolds to be used as delivery vehicles for cells and growth factors. Varying the collagen or the GAG concentrations in the scaffolds affects the osteogenic performance of the scaffolds using MC3T3 osteoblastic cells in-vitro without affecting the structural properties of the scaffolds (Tierney, Jaasma et al. 2008). A limiting factor in using CGAG scaffolds in bone tissue engineering is their poor mechanical properties. Hence, attempts to improve CGAG scaffolds have been made by using cross linking techniques (Tierney, Jaasma et al. 2008; Haugh, Jaasma et al. 2009; Tierney, Haugh et al. 2009). Cross linking aims to improve scaffold's mechanical properties by creating new linking bonds between collagen fibres. This can be done in a number of ways including; chemically using agents such as 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) which creates "cross-liked" bonds between carboxylic acid groups on the collagen polymer; or by physical methods such as heat in dehydrothermal (DHT) cross linking techniques. Increasing the collagen content and using EDAC and DHT cross linking promotes enhanced mechanical and biological properties of the scaffold making it more attractive for use in bone tissue engineering. (Haugh, Jaasma et al. 2009) However, even with these improvements, the scaffolds still have a relative lack of stiffness and strength that may present limitations for clinically applicable bone tissue engineering at some anatomical sites. In this thesis we have investigated these scaffolds in a non-load bearing anatomical site in a rat model.

COMPOSITE SCAFFOLDS

Despite the advances that have been made with both natural polymers and ceramics as bone graft substitutes no single material has proved ideal. Natural polymers show excellent biological performance whilst having poor mechanical properties. Ceramics have mechanical strength similar to bone whilst exhibiting a brittle behaviour and degrading poorly. Composite materials are usually fabricated with one material as a form giving matrix and the other material as reinforcing support. Generally, the reinforcing material is stiffer and mechanically stronger than the surrounding matrix.

Various combinations of ceramic and polymer scaffolds have been tried in order to develop an ideal scaffold for bone tissue engineering. Composite scaffolds of PLLA and HA, and PLGA and HA have shown significantly increased mechanical properties compared to pure polymer foams (Zhang and Ma 1999). PLGA combined with bioactive glass particles has shown an increase in the compressive modulus compared to pure PLGA scaffolds (Maquet, Boccaccini et al. 2004).

Collagen based composite scaffolds have recently been developed, with initial promising results. Composite scaffolds containing a collagen matrix and mineral support have been shown to permit osteogenic differentiation of human bone marrow MSCs with significantly better results than pure collagen scaffolds (Bernhardt, Lode et al. 2008). Octacalcium phosphate/collagen composite scaffolds have shown better healing of a critical sized cranial rat defect compared to pure collagen scaffolds and pure octacalcium phosphate

scaffolds loaded with bone morphegenic protein-2 (Kamakura, Sasaki et al. 2006).

However, scaffolds permeability, porosity and pore structure are highly dependent on the amount of material that is within the scaffold and increasing the amount of reinforcing particles will lead to a reduction in porosity. Furthermore, the distribution of the reinforcing particles plays an important role in the local mechanical properties of the scaffolds. The pore structure has been found to become more irregular and the particles less well distributed throughout the matrix as the percentage of particles was increased in bioglass- Porous poly(alpha-hydroxyacid scaffolds (Maquet, Boccaccini et al. 2004). The challenge in developing a mineralised collagen scaffold is to maintain desirable scaffold porosity and pore size whilst improving mechanical properties.

Aside from improved physical and mechanical properties, mineralised collagen scaffolds have other potential advantages. The mineral component is felt to facilitate bone formation (osteoconduction) and potentially induce new bone formation (osteoinduction). Furthermore, improved biocompatibility over pure collagen scaffolds has been reported (Liao, Wang et al. 2005). From a practical surgical perspective, these scaffolds can easily be fashioned using a scalpel at the time of implantation into the desired shape for defect to be repaired. Avoiding the need for complex pre-op planning for patient specific implants, which are often required with pure ceramic materials.

Our laboratory has recently investigated the introduction of a calcium phosphate mineral phase into the collagen scaffolds that have been typically used, with the aim of maintaining scaffold pore size and porosity. A number of mineralisation techniques have been investigated. Initially, a collagen hydroxyapatite scaffold was developed using an immersion technique whereby a collagen scaffold was soaked in simulated body fluid (SBF) (Al-Munajjed, Plunkett et al. 2009). SBF is an artificial acellular fluid that has inorganic ion concentrations similar to those of human extracellular fluid. Subsequent work has developed an optimised collagen-calcium phosphate (CCP) scaffold, manufactured by immersing pure collagen scaffolds in sodium ammonium hydrogen phosphate (NaNH(4)HPO(4)) followed by calcium chloride (CaCl(2)). These scaffolds have increased mechanical properties over pure collagen scaffolds (90kPa V 0.3kPa) whilst maintaining a high level of porosity at 92% (Al-Munajjed and O'Brien 2009). However, these scaffolds have not been tested in an in-vivo model to date. These scaffolds were thus investigated as part of this thesis.

1.6 MESENCHYMAL STEM CELLS IN BONE TISSUE ENGINEERING

The choice of cell type used in any tissue engineered construct has an important influence on the final tissue type. In the field of bone tissue engineering, the most obvious cell to choose would be autologous osteoblasts. However, osteoblasts are difficult to culture, requiring a long culture period, and are not readily available in most clinical scenarios. Therefore, investigations have focused on the use of stem cells. Stem cells, both embryonic and adult, are characterised by their easy ability to replicate

and ability to differentiate into an array of specialised cell types and have vast potential in the field of tissue engineering.

Embryonic stem cells, isolated from the inner cell mass of blastocysts, despite their desirable pluripotent nature have encountered ethical and legislative barriers to their use (Langer and Vacanti 1993). Adult stem cells or progenitor cells are therefore more widely used in current tissue engineering research, showing promising results in a number of potential clinical areas. They are capable of regenerating themselves throughout the whole life of the organism and can differentiate into specialised cells that make up the tissues and organs of the body through several intermediate cell stages including progenitor cells, precursor cells and final cell type. Differentiating cells reduce their proliferative capacity and become designated to differentiation along that specific lineage (Caplan and Bruder 2001).

Several adult stem cell types, including haematopoietic stem cells (HSC), neural stem cells, mesenchymal stem cells (MSC) and epidermal stem cells have been shown to be capable of self-renewal and differentiation (Verfaillie 2002). Mesenchymal stem cells (also termed multipotent stromal cells) are multipotent and have the ability to differentiate into several cell types; chondrocytes (Johnstone, Hering et al. 1998), osteoblasts (Jaiswal, Haynesworth et al. 1997) , myoctes, adipocytes (Pittenger, Mackay et al. 1999) and most recently beta pancreatic islet cells (Chen, Jiang et al. 2004). MSCs are isolated from bone marrow and separated from red blood cells and

hematopoietic progenitors using their natural adherence to tissue culture plastic.

Promising results have been recently been reported in the field of bone tissue engineering for mesenchymal stem cells seeded onto various scaffold types (Dyson, Genever et al. 2007; Mandal and Kundu 2009). Furthermore, the scaffold itself may affect the MSCs. MSCs seeded onto glass-ceramic scaffolds have demonstrated good cell attachment, proliferation and the expression of osteogenic markers in the absence of osteogenic supplements (Dyson, Genever et al. 2007).

MSCs seeded on different scaffolds have also shown promising results in several in-vivo studies where they have been implanted into critical sized cranial and mandible defects in animal models (Mankani, Kuznetsov et al. 2006; Mankani, Kuznetsov et al. 2006) as well as long bone defects in humans (Quarto, Mastrogiacomo et al. 2001). The first investigators found MSCs on a collagen carrier demonstrated healing of a critical sized mouse calvarial defect (Mankani, Kuznetsov et al. 2006). The new bone and the union to the adjacent host bone were found to be of poor quality. However, with the addition of HA/ tricalcium phosphate particles, enhanced new bone formation was observed. HA scaffolds seeded with autologous bone marrow stromal cells, have been implanted in a small series of human patients (4-8cm tibial defects in 4 patients) resulting in good integration into the host bone, however, the scaffolds showed no signs of degradation (Quarto, Mastrogiacomo et al. 2001).

The ability of MSCs to differentiate depends on various factors, including cell age, density and the presence of certain soluble differentiation factors, which induce differentiation along a particular lineage. The glucocorticoids and TGF-β super family including bone morphogenetic proteins (BMP) are known to play an important regulatory role and induce complete osteogenic differentiation (Aubin, Liu et al. 1995) and are therefore widely used. At present, in in-vitro conditions, the glucocorticoid dexamethasone is used in conjunction with β-glycerophosphate and ascorbic acid-2-phosphate to induce osteogenic differentiation (Jaiswal, Haynesworth et al. 1997; Simmons, Matlis et al. 2003; Wang, Shelton et al. 2003). Dexamethasone is responsible for the up-regulation of bone specific proteins and enzymes, β-glycerophosphate acts as a substrate for the bone forming enzyme alkaline phosphatase and ascorbic acid is involved in the transformation of pro-collagen to collagen. In addition, other molecular factors are proposed to have a role in the induction of osteogenesis. Interleukin-6, independent of dexamethasone (Liu, Aubin et al. 2002); platelet derived growth factor BB (Cassiede, Dennis et al. 1996) and Triodothyronine(Ishida, Bellows et al. 1995) are all suggested to play a role in directing differentiation down a osteogenic pathway.

Aside from the above mentioned chemical factors, the physical environment influences the success of stem cells seeded onto scaffold materials. Bioreactors may be used to encourage cell infiltration into the scaffold as well as good nutrient flow into and waste flow out of the scaffold centre (Jaasma and O'Brien 2008; Jaasma, Plunkett et al. 2008). However, the most

important role of bioreactors is their ability to stimulate cells to differentiate down specific cell lineages. In general, bioreactors vary widely in design and function, depending on the type of stimuli to be applied. Currently, fluid shear compression, tension and hydrostatic pressure are used to stimulate cells. Cyclic mechanical strain has been demonstrated to be effective at inducing chondrogenesis (Angele, Yoo et al. 2003), as has pulsatile radial stress for myocytes (Niklason, Gao et al. 1999). Mechanical strain has also been shown to affect osteoblastic activity in-vitro (Jaasma and O'Brien 2008; Jaasma, Plunkett et al. 2008).

Potential problems of MSC seeded tissue engineered constructs do exist. Stem cells have a tendency to congregate at the periphery of scaffolds, leading to a lack of nutrient delivery to and waste removal from the centre of the construct (Kelly and Prendergast 2004; Farrell, van der Jagt et al. 2008). This is likely caused by insufficient neovascularisation during host – construct integration. Furthermore, in-vitro culture of a scaffold for several weeks prior to implantation in-vivo can lead to an extensive extracellular matrix deposition at the periphery of the construct. This "encapsulation" inhibits blood vessel infiltration in-vivo and seals the pores in the circumference of the scaffold surrounding an avascular area in the scaffold centre preventing further cellular infiltration. These issues are likely to pose further problems as implanted scaffolds become bigger as animal models expand from small rodents to larger animals.

1.7 SCAFFOLD DEGRADATION

The manner by which a scaffold degrades in-vivo is of crucial importance in the long term success of a tissue-engineered construct. The rate of degradation may affect many cellular processes including cell growth, tissue regeneration, and host response (Mikos, McIntire et al. 1998). The ideal rate of degradation should not exceed the rate of new bone formation and the reduction of strength of the implant should closely match the increase in tissue strength, otherwise the stresses could be transferred to the healing bone, which could be detrimental for bone healing (Liao and Cui 2004). Additionally, degradation is important if collagen scaffolds are to have potential as drug releasing devices, with collagen bound molecules being released in tandem with collagen degradation (Boateng, Matthews et al. 2008).

As collagen scaffolds degrade in vitro, their mechanical properties deteriorate. Previous work has indicated that the resistance of collagen to degradation by collagenase increases with the cross-linking density of collagen (Weadock, Miller et al. 1996; Pek, Spector et al. 2004). Aside from cross linking, the degradation rate of collagen has also shown to decrease significantly following the incorporation of GAG chains into the collagen network (Pek, Spector et al. 2004). However, the effect of introducing a mineral phase on the degradation characteristics of composite collagen scaffolds is poorly understood, and has not been reported in-vitro or in-vivo for the scaffolds developed in our laboratory and has been investigated robustly in this thesis.

During the development and assessment of clinical utility of any new scaffold material degradation ought to be thoroughly studied. An understanding of the effect that the scaffold's constituent materials have on degradation permits the tailoring of the degradation rate to that required for different tissue types in different clinical locations. Furthermore, appropriate knowledge of the materials characteristics is necessary for regulatory approval with degradation an important outcome of the characterization required by the Drug Administration (FDA) prior to regulatory approval *((FDA) 2007).*

COLLAGEN SCAFFOLD DEGRADATION

Collagen fibrils are degraded by a process involving water absorption which results in swelling of the collagen fibril followed by enzyme penetration and digestion of collagen linkages. The specific ability to cleave collagen at its undenatured helical at physiological pH and temperature regions is unique to a small group of enzymes termed collagenases (Harrington 1996). This collagenase digestion does not result in specific amino acids, but results in fragments that are further broken down to individual amino acids by other general proteinases (Mallya, Mookhtiar et al. 1992). Collagenases are released by osteoclasts, during bone re-modelling, in order to break down the collagenous network in bones. Therefore any collagen-based scaffold implanted into bone is likely to be exposed to these enzymes with subsequent biodegradation. However, degradation with collagenase tends to occur over a short period of time, with some scaffolds fully degrading within 3 hrs in an in-vitro study (Wahl, Sachlos et al. 2007).

In-vivo degradation of collagen scaffolds is more complex. The implant is infiltrated by inflammatory cells, which release protein-cleaving enzymes. Up to four different classes of proteinases may be involved which are either stored within cells or released when required namely; Cystein and aspartic proteinases as well as serine and collagenases (Shingleton, Hodges et al. 1996). Furthermore, non-enzymatic degradation mechanisms by hydrolysis, participate in the breakdown of collagen (Okada, Hayashi et al. 1992). However, collagenases are thought to be predominantly responsible for the degradation of the extracellular matrix. In-vivo degradation of tissue engineered collagen scaffolds is generally rapid but is affected by the characteristics of the scaffold, in particular the addition of a cross linking step to the manufacture process (van Wachem, van Luyn et al. 1994; Weadock, Miller et al. 1996). The addition of glycosaminoglycan (GAG) carbohydrates into collagen chains in order to improve their mechanical properties has also been shown to suppress in vivo degradability (Yannas 1992).

1.8 COLLAGENASES IN BONE BIOLOGY

Matrix metalloproteinases (MMPs) constitute a family of zinc dependent proteinases which have essential roles in degradation of ECM components. A subfamily of the MMPs (MMP-1, MMP-2 and MMP-13) exists which demonstrate the ability to cleave the triple helical structure of collagen (Vincenti and Brinckerhoff 2002). In recognition of this collagenolytic ability these enzymes are also known by the synonyms Collagenase-1, Collagenase-2 and Collagenase-3 respectively. MMP-13 is implicit in a

number of pathological disease processes including malignancy, osteoarthritis, rheumatoid arthritis and periodontal disease (Baker, Edwards et al. 2002; Kiili, Cox et al. 2002; Leonardi, Talic et al. 2007; de Aquino, Guimaraes et al. 2009), and can cleave type I and II collagens (Lovejoy, Welch et al. 1999). MMP-13 gene expression is induced by various cell types including chondrocytes, endothelial cells, osteoblasts, fibroblasts and peri-osteoclastic cells (Solis-Herruzo, Rippe et al. 1999; Mengshol, Vincenti et al. 2001; Zaragoza, Soria et al. 2002; Varghese and Canalis 2003; Andersen, del Carmen Ovejero et al. 2004). However, MMP-13 is also required in non-pathological physiological processes such as endochondral bone development. Fracture healing and bone remodelling have been shown to be impaired in MMP-13 deficient mice (Kosaki, Takaishi et al. 2007). MMP-13 mRNA has been demonstrated in periosteoclastic cells and MMP-13 protein in the subosteoclastic resorption zone. Further evidence that MMP-13 has important key functions in the formation and remodelling of bone is provided by the fact that its expression in osteoblasts is decreased by several bone morphogenetic proteins (BMPs -2, -4 and -6) and increased by parathyroid hormone (Leeman, Curran et al. 2002). The presence of MMP-13 at sites of bone healing and its collagenolytic function has led us to hypothesise a role for this enzyme in the degradation of our collagen (type I) based scaffolds and has therefore been investigated in this thesis.

1.9 HOST IMMUNE RESPONSE TO COLLAGEN SCAFFOLDS AND TISSUE ENGINEERED COLLAGEN CONSTRUCTS

The host immune response to biologic scaffolds is complex and determined by the nature of the biologic scaffold material and by the manufacturing methods utilised. One study which analysed the host response to five different mammalian extracellular matrix derived products identified a universal immune response characterised by an intense mononuclear cell infiltrate. However, the long term remodelling response, was varied between encapsulation, chronic inflammation, fibrosis, scarring to the formation of site appropriate remodelled tissue. There were also differences in the intensity of the immune response, the location of the response and it's time course (Valentin, Badylak et al. 2006). In the investigation of such host immune responses, it has emerged that histological assessment is insufficient and additional techniques such as immunohistochemistry are required, to define the cell types present.

The host response involves both the innate and acquired immune system with the response affected by a number of factors including; the intended clinical application, the source of the raw material for the scaffold and processing steps involved in the product manufacture (Badylak and Gilbert 2008).

The role of the T lymphocyte mediated cellular immune response has been poorly studied in biologic tissue engineered devices. However, their role in the immune response to xenografts is widely studied and elucidated by researchers in the field of organ transplatation. Th1 (Helper T cell type 1)

lymphocytes produce cytokines such as interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-β leading to macrophage activation, stimulation of complement fixing Ab isotypes and differentiation of CD8+ cells to a cytotoxic phenotype. This response is associated with transplant rejection (Strom, Roy-Chaudhury et al. 1996). Meanwhile, Th2 lymphoctes produce IL-2, IL-5, IL-6, and IL-10 cytokines that do not activate macrophages and lead to production of non-complement fixing istotypes. Activation of the Th2 pathway is associated with transplant acceptance (Piccotti, Chan et al. 1997).

Macrophages are activated in response to tissue damage or infection, causing an increase in the production of cytokines, chemokines, and other inflammatory molecules to which they are exposed. In wound healing and at sites of inflammation mononuclear cells follow neutrophils to the site, where they phagocytose cellular debris and foreign material prior to eventually exiting from the site of inflammation (Kumar, Abbas et al. 2005). Recently, macrophage phenotype has been characterised according to distinct functional properties, surface makers, and the surrounding cytokine profile. Thus macrophages can be labelled as either of M1 or M2 phenotype (**Figure 1.5**). M1 type macrophages appear to be mainly concerned with the acute inflammatory response and are characterised by CCR7 and CD80+ cell wall markers (that can be identified by specific monoclonal antibodies using Immunohistochemical techniques) in rats (differences between species exists), the production of large amounts of nitric oxide and the pro inflammatory cytokines IL-12 and TNFα. Meanwhile M2 cells possess the ability to facilitate tissue repair and regeneration, via the expression of IL-10

and TGF-β, production of arginase, inhibition of pro-inflammatory cytokines, debris scavenging and the promotion of angiogenesis. M2 cells are characterised by the expression of the CD163 surface marker (Anderson and Mosser 2002; Mosser 2003; Gordon and Taylor 2005; Mantovani, Sica et al. 2005) (**Figure 1.6**).

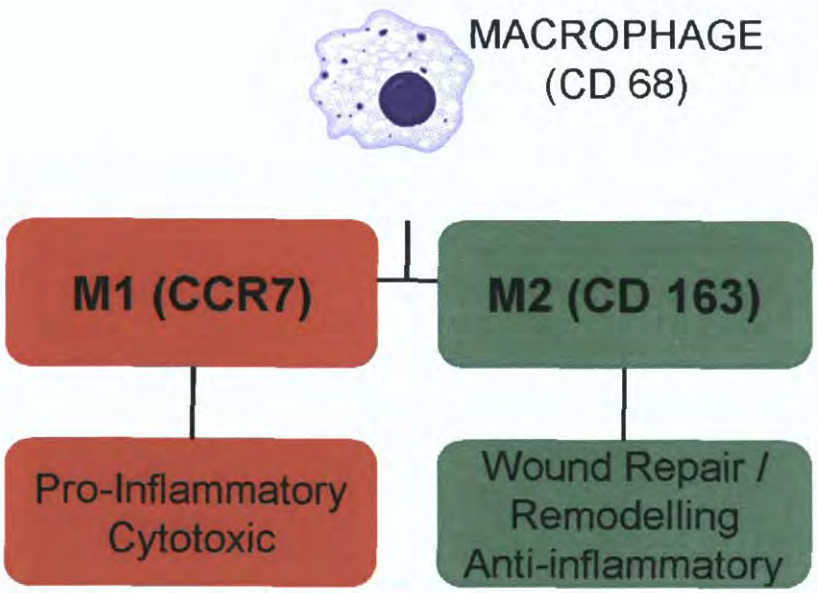

Figure 1.5. *The M1/M2 phenotypes of macrophages and their associated role in the host immune response.*

The effect of macrophage phenotype on remodelling of tissue engineered constructs is currently poorly investigated and understood, but is known to be affected by both the presence of cellular material and manufacturing techniques. In one study using an implanted ECM scaffold, those specimens containing a predominantly cellular component were found to elicit a predominantly M1 type macrophage response, with subsequent deposition of dense connective tissue and / or scarring, whilst constructs without any cellular components were associated with a M2 type response and associated

active remodelling (Brown, Valentin et al. 2009). MSCs are reported to be both immunoprivileged (not targeted by MHC-mismatched immune cells which traditionally mediate immunorejection in transplantation) and immunomodulation (demonstrating an immunosuppressive response), however the latter appears to be lost after host implantation (Liu, Kemeny et al. 2006). Scaffold crosslinking may also affect the response, carbodiimide crosslinking of small intestinal submucosa (SIS) scaffolds has been shown to be associated with an M1 phenotype response as opposed to an M2 predominant response for non-cross linked scaffolds in a rat model (Badylak, Valentin et al. 2008).

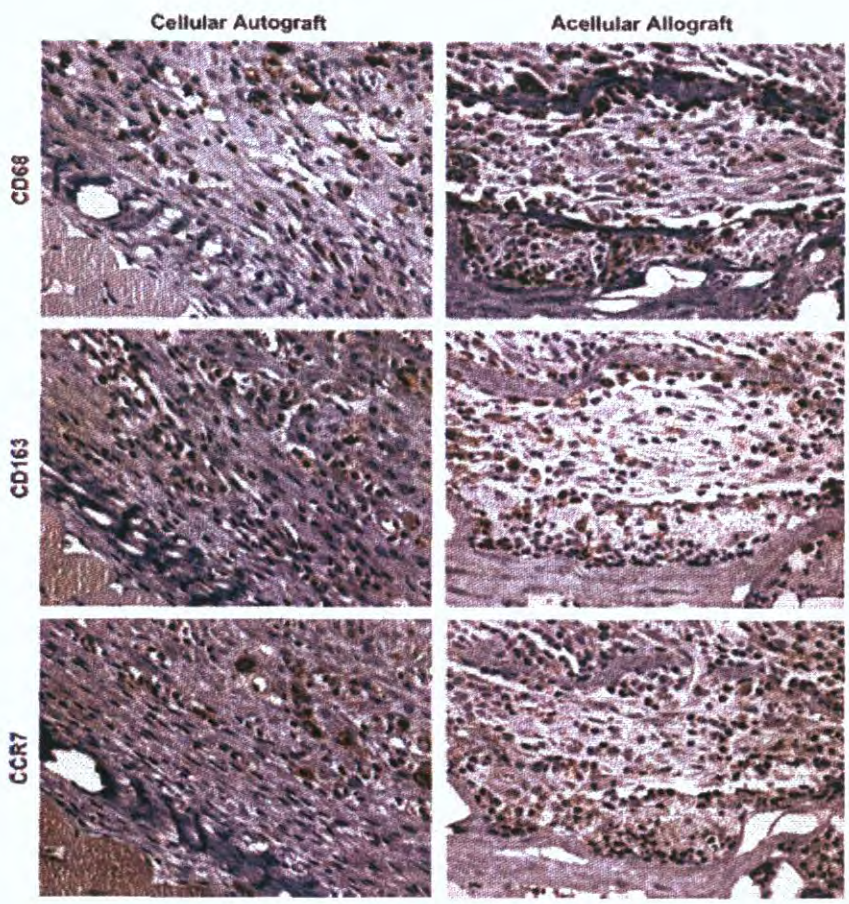

Figure 1.6. *Example of immunohistochemical staining for a pan-macrophage marker (CD68), M2 phenotype macrophages (CD163) and M1 phenotype macrophages (CCR7) in cellular and acellular grafts (Brown, Valentin et al. 2009).*

In bone biology, it is long established that macrophages play an important role in the initial inflammatory phase of fracture repair, infiltrating the fracture haematoma secreting cytokines and phagocytising degenerated cells and other cellular debris (Schindeler, McDonald et al. 2008). It would seem likely that macrophages play an important role in the host response to a surgically created defect and implantation of tissue engineered scaffold constructs. However, to date no work has been published on the macrophage response and phenotype in the host response to collagen based scaffolds designed for bone tissue engineering such as those used by our group.

1.10 THESIS OBJECTIVES

GENERAL OBJECTIVE

The objective of this work was to determine how the addition of a calcium phosphate mineral phase to a collagen-based scaffold, designed for use in bone tissue engineering, affects the in-vitro and in-vivo scaffold degradation characteristics in addition to the rate of tissue healing as assessed by new bone formation and host immune response in a rat calvarial model, and to determine if the culture with mesenchymal stem cells affects these characteristics.

SPECFIC AIMS

IN-VITRO DEGRADATION CHARACTERISTICS OF COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS

- To determine if the addition of a calcium-phosphate based mineralisation phase in a collagen scaffold affects its degradation characteristics.
- To determine the change in mechanical properties of collagen and mineralised collagen scaffolds over the passage of time in different degradation media.

IN-VIVO ASSESMENT OF SCAFFOLD DEGRADATION CHARACTERISTICS, RATE OF TISSUE HEALING INCLUDING NEW BONE FORMATION AND HOST IMMUNE RESPONSE TO IMPLANTED TISSUE ENGINEERED COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS

- To quantitatively determine the rate of new bone formation and degradation characteristics when collagen and mineralised collagen scaffolds are implanted in a rat calvarial defect.

- To determine the effect of scaffold pre-culture with mesenchymal stem cells on the rate of new bone formation and degradation characteristics of the scaffold.

- To determine the phenotype of macrophages involved in the normal host immune response to implanted collagen scaffolds (both scaffolds alone and those tissue engineered with mesenchymal stem cells) and to assess whether the M2 phenotype response (wound healing) is associated with scaffold degradation and new bone formation.

CHAPTER 2 - MATERIALS & METHODS

2.1 IN VITRO DEGRADATION CHARACTERISTICS OF COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS

2.1.1 SCAFFOLD MANUFACTURE

PURE COLLAGEN SCAFFOLDS

All scaffolds used in this study were initially produced from collagen slurry. Type I bovine collagen (Integra Life Sciences, Plainsboro, NJ –Received under the terms of a materials transfer agreement) and 0.05M glacial acetic acid were blended together at 15,000 rpm using an overhead blender (Ultra Turrax T18, IKA Works Inc., Wilmington, NC). Blending was carried out in a reaction vessel, which was maintained at 4°C using a circulation cooling system (WKL 230, Lauda, Germany). The resulting collagen slurry contained 0.5% (w/v) collagen. The slurry was degassed in a vacuum desiccator to remove trapped air bubbles after blending.

The slurry was freeze-dried following the protocol developed by O'Brien *et al.* (2004) (O'Brien, Harley et al. 2004). Slurry was pipetted (67.25 ml) into a stainless steel tray (12.7 x 12.7 cm, grade 304 SS). The tray was placed onto the freeze-dryer shelf (Advantage EL, VirTis Co., Gardiner, NY) and the freezing cycle was started. A freeze drying recipe with a freezing temperature (T_f) of -40°C (Appendix I), produced by a constant cooling technique, was used, based on previous work to identify optimum collagen scaffold

characteristics for tissue engineering (O'Brien, Harley et al. 2004; O'Brien, Harley et al. 2005).

COLLAGEN GLYCOSAMINOGLYCAN SCAFFOLDS

collagen-glycosaminoglycan (CollGAG) scaffolds were fabricated using a similar freeze-drying technique (O'Brien, Harley et al. 2004; Tierney, Jaasma et al. 2008). Briefly, a CollGAG suspension was produced in a solution of 0.05M acetic acid using 7.2 g (1% w/v) type I collagen, isolated from bovine tendon (Integra Lifesciences, Plainsboro, NJ) combined with 0.32 g (0.044% w/v) chondroitin-6-sulphate, isolated from shark cartilage (Sigma-Aldrich, Germany). The slurry was blended at a temperature of 4°C to prevent denaturation of the collagen fibres as a result of the heat generated by the mixing process. The resulting solutions were degassed in a vacuum to remove any air and subsequently freeze-dried at -40°C using a constant cooling technique (O'Brien, Harley et al. 2004).

COLLAGEN CALCIUM PHOSPHATE SCAFFOLDS

Collagen-calcium phosphate (CCP) scaffolds were fabricated as previously described using a freeze-drying technique (O'Brien, Harley et al. 2004) and a bi-phasic immersion technique (Al-Munajjed and O'Brien 2009). Briefly, a pure collagen suspension was mixed using type I collagen, isolated from bovine tendon (Integra Lifesciences, Plainsboro, NJ) in a solution of 0.05M acetic acid. The slurry was blended at a temperature 4°C, degassed under vacuum and freeze-dried at -40°C using a constant cooling technique (O'Brien, Harley

et al. 2004). Calcium chloride ($CaCl_2$) and ammonium sodium hydrogen phosphate ($NaNH_4HPO_4$) (Sigma Aldrich, Germany) solutions at a 0.1M concentration were prepared by mixing in Tris buffer at a pH of 7.4 (50 mM Tris, 1% NaN_3) according to Yaylaoglu *et al.* (Yaylaoglu, Korkusuz et al. 1999). Samples with a diameter of 7mm and a height of 4mm were cut from collagen sheets with a punch and pre-hydrated in PBS. The scaffolds were then mineralized using stepwise immersions in the chemical solutions, $NaNH_4HPO_4$ and $CaCl_2$. The scaffolds were immersed in $NaNH_4HPO_4$ for 22 hours, followed by 22 hours in $CaCl_2$. This procedure was then repeated.

2.1.2 SCAFFOLD CROSS LINKING

Both the pure collagen and collGAG scaffolds underwent dehyrdrothermal (DHT) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) crosslinking in order to improve their mechanical properties towards that required for bone tissue engineering. In addition, the DHT process acts as a sterilisation step as is necessary for any surgically implanted construct ((EMEA) 2000; Geiger, Li et al. 2003).

DEHYDROTHERMAL CROSS LINKING

DHT treatment was carried out by placing the scaffold sheets in an open aluminium foil packet, placed upright inside a vacuum oven (Vacucell 22, MMM, Germany) under a vacuum of 0.05 bar. Exposure period was 24hr at a cross linking temperature of 105°C. Following the procedure, the foil package was sealed and placed in an airtight box with a desiccant pack.

EDAC (1-ETHYL-3-(3-DIMEHTYLAMINOPROYPL) CARBODIIMIDE)

Cylindrical samples (9.525mm (3/8 inch) diameter, 4 mm height) were cut from the scaffolds prior to cross linking, using a custom designed hand punch. Samples were then pre-hydrated in phosphate buffered saline (PBS, Sigma-Aldrich, Germany) prior to EDAC cross linking. Scaffolds were cross linked using a concentration of 6 mmol of EDAC per g of collagen. This concentration was chosen based on the results of a previous study (Olde Damink, Dijkstra et al. 1996). EDAC was prepared in distilled water and the samples were cross linked in a 24 well plates, containing 2mls of EDAC solution per well, for 2 hrs at room temperature. A molar ratio of 2.5 mol NHS/mol EDAC was used for all EDAC cross linking. Scaffolds were then transferred into separate wells containing PBS and washed on a plate rocker for 30 minutes. PBS washing was repeated twice.

2.1.3 SCAFFOLD DEGRADATION

STANDARD DEGRADATION

Following manufacture, specimens were weighed dry (Wo) using a microbalance (Mettler Toledo, MX5, Schwerzenbach, Switzerland). It was not possible to perform a dry weight on the CCP scaffolds as they are wet post their immersion manufacture process. For these scaffolds, a sample of scaffolds were freeze-dried and weighed to give an average dry weight (Wo). Immediately following the EDAC cross linking process (including washing in PBS), eight specimens per time point were placed in 2ml of Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich Company Ltd, Aryshire, UK) in

individually labelled wells of a 24 well plate. The plates containing the specimens were then incubated at body temperature (37.4 °C) and an environment of 5% CO^2.

At each time point 3 samples were removed from the PBS, dipped in distilled water 10 times, placed on a 24 well plate and lyophilized using a freeze drier. Having been removed from the freeze drier, specimens were re-weighed using the microbalance (Wt). Percentage degradation was calculated using the following formula (Liao and Cui 2004).

$$\text{Weight loss (\%)} = \frac{(Wo - Wt) \times 100}{Wo}$$

Each result was the average of three parallel measurements, expressed as mean ± standard deviation.

COLLAGENASE DEGREDATION

In vitro degradation of the scaffolds was also performed using a bacterial collagenase type I (Enzyme commission number 232-589-9) obtained from Clostridium histolyticum (Sigma Aldrich, St. Louis, MO, USA) with a collagenase activity of 345 units/mg solid (collagen). The enzyme was dissolved in PBS with 0.5mM of Calcium Chloride (Sigma). The concentration of Collagenase was chosen experimentally based on a number of trial experiments observing the degradation of similar scaffolds in different concentrations of Collagenase. A Collagenase concentration of 0.05 mg/ml PBS was found to result in significant but not complete degradation of all the scaffolds at 6 hrs. This concentration was similar to the lowest concentration

used by Park et al. (Park, Lee et al. 2003) Calcium chloride was added as collagenase is activated by four gram atom calcium (Ca^{2+}) per mole enzyme and (Schomberg and Salzmann 1991). A trial experiment had demonstrated no degradation of collagen and collagen GAG scaffolds when immersed in a collagenase solution without the addition of $CaCl_2$. A concentration of 0.5mM $CaCl_2$ was used as this has been reported to be the optimum for tissue dissociation by the enzyme manufacturer.

Immediately following the EDAC cross linking process (including washing in PBS), eight specimens per time point were placed in 2ml of the collagenase/PBS solution in individually labelled wells of a 24 well plate. The plates containing the specimens were then incubated at body temperature (37.4 °C) and an environment of 5% CO_2.

At each time point, collagenase activity was inhibited by the addition of 0.2ml of 0.25 M ethylenediaminetetraacetic acid (EDTA) to each well (Wahl, Sachlos et al. 2007). Three samples were removed from the PBS, washed with distilled water and lyophilized using a freeze drier (-40°C recipe). If there was any delay in placing the specimens in the freeze drier, they were placed in a freezer at -20 °C in the interim (to further ensure inhibition of enzyme activity). Having been removed from the freeze drier, specimens were re-weighed using the microbalance (Wt). Percentage degradation was calculated using the same equations as for the standard degradation group. One sample was placed in formalin for 1hr prior to tissue processing and 4 samples underwent immediate mechanical testing.

2.1.4 MECHANICAL TESTING

Wet compressive mechanical testing was used to determine if the mechanical properties of the scaffold changed with increased exposure to the various degradation media. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) attached to a PC and fitted with a 5-N load cell. Testing was performed on four of the eight samples incubated at each time point. Samples from the PBS only group were tested directly from this media. Whilst the samples exposed to Collagenase were pre-hydrated in PBS, following inhibition of the enzyme by EDTA and washing with distilled water. Prior to mechanical testing these samples had their diameter measured using digital callipers in three separated directions, these measurements were used to give a mean radius (r). The height (h) of the specimen was measured by the mechanical testing machine as the distance between the two compression plates with the scaffold material touching both plates.

Unconfined compressive testing were conducted at a strain rate of 10%/min, with impermeable, un-lubricated platens. The modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain, avoiding the less stiff toe region of the stress-strain curve. Each result was the average of the four parallel measurements, expressed as mean ± standard deviation.

2.1.5 IMAGING

At each time point (after the arrest of collagenase activity) digital photographs (Canon EOS 350D) were taken of specimens in order to make a visual comparison of the in-vitro scaffold degradation process.

2.2 IN VIVO ASSESMENT OF SCAFFOLD DEGRADATION CHARACTERISTICS AND HOST IMMUNE RESPONSE TO IMPLANTED COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS WITH AND WITHOUT MESENCYMAL STEM CELL SEEDING

2.2.1 EXPERIMENT DESIGN

In order to assess the in vivo rate of new bone formation, host tissue response to the scaffolds and scaffold degradation, histomorphometry and immunohistochemical analysis was performed on specimens implanted into a calvarial defect with co-workers Dr. Frank Lyons and Dr. Amir Al-Munajjed. This study was approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, and an animal license was granted by the Irish Government Department of Health. 48 young adult male Wistar rats (mean weight 375g; range 360-395g) were utilised for this study. Following creation of a 7mm trans-osseous calvarial defect either, a CGAG scaffold, CCP scaffold, or no scaffold was implanted into the defect. Half of each group was selected at random for sacrifice at either 4 or 8 weeks. Half of each scaffold group were pre seeded with mesenchymal stem cells.

2.2.2 MESENCYMAL STEM CELL CULTURE AND SEEDING

Mesenchymal stem cell harvesting, culture and scaffolds seeding was performed in adherence to an in house laboratory protocol developed from extensive experience with MSCs and collagen based scaffolds (Farrell, O'Brien et al. 2006). Briefly, three-month-old Wistar rats (300–350g) were sacrificed by CO_2 asphyxiation in accordance with local guidelines and ethical

approval by a licensed veterinary surgeon. The femur was separated from the tibia and placed in sterile pre-warmed Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, Poole, United Kingdom) supplemented with 10% foetal bovine serum, 100U/mL penicillin/streptomycin, 2mM Glutamax, 1mM L-glutamine, and 1% non-essential amino acids. The femur and tibia were cut at both epiphyses, and marrow was flushed into a 50mL tube using 5mL supplemented DMEM and a 25-gauge needle. The suspension was centrifuged (650g, 5min, 20°C), re-suspended in 10mL of DMEM and passed sequentially through 16-, 18-, and 20-gauge needles. The suspension was passed through a 40m nylon mesh and incubated for 30min in a tissue culture flask in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The supernatant was removed and split between two T75 flasks; following a 24h period, the culture medium was replaced to remove non-adherent cells. Upon reaching 80–90% confluence, the cells were passaged and replated into two T175 tissue culture flasks. The medium was replaced every 3-4 days, and cells were maintained in a humidified atmosphere of 95% air/5% CO2 at 37°C until passage number 5. Cells were rinsed with PBS and detached with trypsin-EDTA (Sigma-Aldrich). The resulting suspension was centrifuged (2000g, 5min at 20°C), re-suspended in 2mL supplemented DMEM, and aspirated through a 20-gauge needle to obtain a single cell suspension of 2.5 million cell/mL.

CollGAG and CCP scaffolds underwent seeding, which involved immersion in a 100μl cell suspension containing 500,000 cells and incubated for 15min in 6-well plates. The scaffolds were then turned over and a further 100μL of cell

suspension was placed onto the opposite scaffold side. After 15min, 5mL of supplemented DMEM was added to each well in order to fully submerge the scaffold. To induce osteogenesis, the scaffold was placed in DMEM supplemented with 0.68nM dexamethasone, 10mM glycerophosphate, and 50μM ascorbic acid. Half of the respective media was replaced every 3-4 days. Scaffolds were cultured for 28 days prior to animal implantation and were maintained in a humidified atmosphere of 95% air/5% CO2 at 37°C.

2.2.3 SCAFFOLD IMPLANTATION AND ANIMAL SURGERY

Under Irish Government licence, all rats were prepared for the surgical procedure by the designated veterinary surgeon. Anaesthesia was induced with intra-peritoneal mixed xylazine hydrochloride 75mg/kg and ketamine hydrochloride 10mg/kg and maintained with isofluorane and oxygen delivered through a facemask. At induction, prophylactic analgesia and antibiotics were given in the form of carprofen 4 mg/kg and enrofloxacin 5 mg/kg delivered sub-cutaneously. The rat was then positioned supine on the operating table and the skin over the head shaved and prepared with 10% aqueous iodine skin solution before draping. A 1.5cm midline sagittal incision was made over the rat calvarium exposing the periosteum which was incised and reflected. Using a dental trephine burr drill (Dentalfarm, Torino, Italy), a 7 mm circular transosseous defect was created in the rat parietal calvarium, lateral to the saggital suture under constant saline irrigation (**Figure 2.1**). The scaffold was implanted into the defect, the periosteum oversewn with 5/0 absorbable monocryl suture (Ethicon, NJ, USA) and the skin closed with skin clips. The rat was then passed back to the veterinary surgeon for routine post-operative

care. All rats were housed in the Biomedical Research Facility at the Royal College of Surgeons in Ireland, Dublin 2, Ireland. Animals were sacrificed, by CO2 asphyxiation, at 4 and 8 weeks post-surgery, whereupon a 20 x 20 mm segment of calvarium containing the defect was removed and placed in labelled formalin jars.

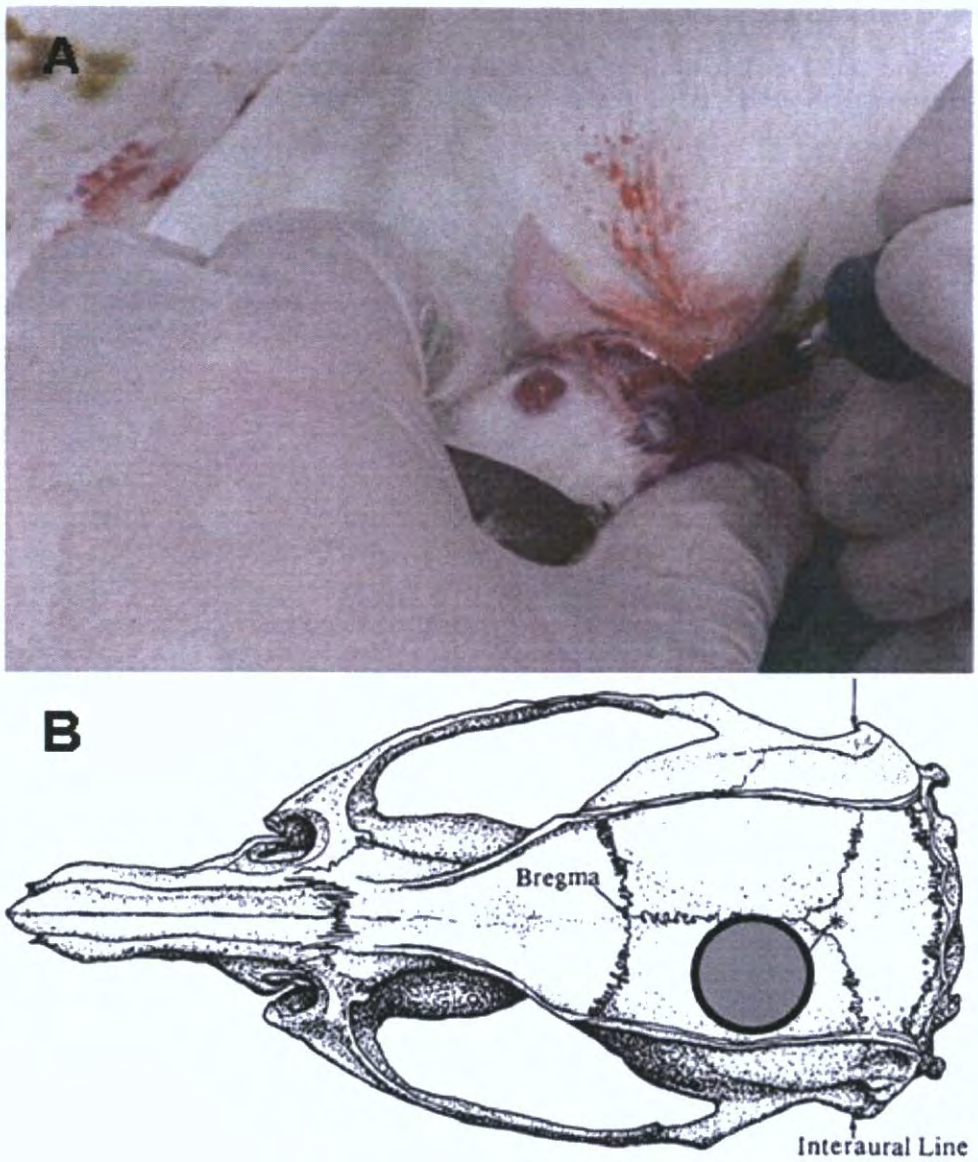

Figure 2.1. *(a) Intraoperative photograph of the 7mm critically sized defect being created in the rat calvarium with a dental burr. Image (b) identifies the defect location in the rat calvarium.*

2.2.4 HISTOLOGY

Following sacrifice, specimens were fixed in formalin prior to decalcification in 10% formic acid for 2 weeks during which period serial plain x-ray was used to monitor the decalcification process. The formic acid was changed every 3 days. Specimens were then embedded in paraffin using an automated tissue processor (ASP 300, Leica Microsystems GmbH, Wetzlar, Germany). Following adequate decalcification, each specimen was bisected and embedded in a single paraffin wax block. The median surfaces were orientated superiorly so that each section represented equal depth from the midline. Six µm coronal sections were prepared using a hand operated rotary microtome (Microsystems GmbH, Wetzlar, Germany). Sections were mounted on L-polysine coated glass slides (Thermo Scientific, Menzel Gmnh & Co KG, Braunshweig, Germany). Sections were then placed in an oven overnight at 60°C.

These sections were dewaxed by immersion in two change of Xylene, rehydrated with graded alcohols (100 to 70%) and stained with haemotoxyoin and eosin using a routine protocol (Appendix I). A representative image of each specimen was acquired and digitized using a microscope attached to a PC (Nikon Microscope Eclipse 90i with Nis Elements software v3.06, Nikon Instruments Europe, The Netherlands). Areas within each image were identified as either 1) host bone, 2) woven bone, 3) inflammatory tissue, 4) osteoid, 5) residual scaffold. Areas were also qualitatively observed for overall defect healing, neo-vascularisation, host-cell penetration and scaffold integration.

2.2.5 HISTOMORPHOMETRY

Quantitative analysis was performed on representative sections from each specimen, an image of these sections was acquired and digitized. The diameter of the defect and percentage of new bone formation for each group were determined by histomorphometry. Image analysis was performed using Nis Elements software v3.06 (Nikon Instruments Europe B.V, Badhoevedorp, The Netherlands). In this way the distance between the two sides of the defect was measured. New bone was identified by magnified inspection of the image and its' area quantified using the image analysis software. The total area of new bone formation was calculated by summing these areas A_{NB}. A percentage new bone formation was calculated by comparing this measure to the total area of the original defect A_T using the following formula.

$$\% \text{ New Bone} = \frac{A_{NB} \times 100}{A_T}$$

In order to assess the amount of scaffold degradation, the area of scaffold remaining (A_s) at each time point was calculated in a similar manner. The area of individual regions of scaffold remaining (not degraded or not replaced by new bone) were measured and summed. The percentage scaffold degradation was calculated using the following formula.

$$\% \text{ Loss of scaffold area} = \frac{(A_T - A_S) \times 100}{A_T}$$

2.2.6 IMMUNOHISTOCHEMICAL STAINING

Specimens were processed in an identical manner for immunohistochemistry as for routine histology. Immunohistochemical analysis was performed in order to assess the inflammatory response to the implantation of cell seeded and non-cell seeded collagen scaffolds, and furthermore to determine the predominant macrophage phenotype in such responses. Staining was also performed in order to detect MMP-13 (Collagenase 3) according to methods recommended for skeletal tissues (Andersen, del Carmen Ovejero et al. 2004). Having been dewaxed with Xylene and rehydrated in descending grades of alcohol (100% to 70%), specimens were then washed in two changes of PBS (Sigma) for 5 minutes twice. A trial experiment revealed that no antigen retrieval step was necessary. Endogenous peroxidise activity was inhibited by incubating the specimens with 3% H_2O_2 (Sigma, diluted in distilled H_20). This step was again followed by rehydration with PBS for five minutes, twice. Specimens were encircled on the slide using a PAP marking pen. Sections were then incubated in 1.5% normal horse serum or normal goat serum, depending on species of origin of secondary antibody, (Vectastain Elite Kit, Vector laboratories, Burlingame, CA, USA) for 20minutes in a humidified chamber at room temperature in order to inhibit non specific binding of the primary antibody. This blocking serum was blotted off and the specimens incubated with primary antibody in a humidified chamber at 4^0C, overnight. The primary antibody was prepared in 1% bovine serum Ibumin (Sigma) in PBS. Each tissue specimen was exposed to antibodies to a pan-macrophage marker (CD 68), an M1 phenotype marker (CCR7), an M2 phenotype marker (CD163) and for MMP13.

Following overnight incubation, the slides were washed twice in PBS and incubated with a biotinylated secondary antibody (Vectastain Elite Kit, Vector) for 30 minutes at room temperature. This antibody was subsequently blotted off and the slides washed twice in BPS prior to the application of the Vectastain ABC reagent (Vectastain Elite Kit, Vector) in a humid chamber for 1 hour at room temperature. Specimens were then washed twice in PBS and once in distilled water for 5 minutes each. In order to detect peroxidise activity, 3,3'-Diaminobenzidine (DAB, 0.7mg/ml; Sigma) was applied to the specimens for 5 to 10 minutes at room temperature in a dark chamber until a satisfactory colour change was achieved. The slides were then rinsed in tap water to inhibit the DAB reaction and lightly counterstained with Harris Haematoxylin stain. Finally slides were dehydrated using a reverse of the previous alcohol steps including an immersion in Xylene prior to coverslipping.

The primary antibodies used for immunohistochemical staining were mouse anti-rat CD 68 (Clone ED1, Serotec, Raleigh, NC, USA) at a dilution o f 1:50, mouse anti-rat CD 163 (Clone ED2, Serotec) at a dilution of 1:50, rabbit anti-CCR7 (Cell applications, San Diego, CA, USA) at a dilution of 1:200 and mouse anti-rat MMP 13 (Ab -3, Clone LIPCO IID1, Neomarkers, Fremont, Ca, USA) at a dilution of 1:100. The secondary antibodies utilised were biotinylated horse anti-rabbit horse anti-mouse IgG (Vector, PK 6101; CD 68, CD 163, MMP 13) at a working dilution of 1:200 and goat anti-rabbit IgG (Vector, PK 6102; CCR7) at a dilution of 1:200. All antibodies were diluted in sterile, filtered PBS (pH 7.4). Antibody dilutions were initially chosen based on

the literature and subsequently adjusted based on trial experiment's staining patterns. (Leonardi, Talic et al. 2007; Brown, Valentin et al. 2009; de Aquino, Guimaraes et al. 2009) Selected specimens underwent the immunohistochemical staining process in the absence of a primary antibody, none of these specimens showed post DAB staining, confirming the absence of inordinate background stain.

In a similar manner to the H&E slides, representative images of the immunohistochemically stained specimens were recorded digitally using Nikon Nis Elements software v3.06 software run on a pc attached to a Nikon Eclipse 90i microscope. Images were taken at x4 to include the host / implant interface with the host orientated to the left side. Higher powered images x20 were take from areas of interest within the field of the original (lower magnification) image.

2.2.7 STATISTICAL ANALYSIS

Data was collated in Microsoft Excel spreadsheets (Microsoft Excel 2003, Microsoft Corp, Redmond WA, USA). One-way analysis of variance (ANOVA) and pair-wise multiple comparison procedures were used to compare data groups using Analyse-it software (v2.04. Leeds, United Kingdom). Dunnette or Tukey contrast tests were used depending on whether a pairwise or control analysis was performed. Error is reported in text and figures as the standard deviation. A probability value of 95% ($p < 0.05$) was used to determine significance.

CHAPTER 3 - RESULTS

3.1 IN VITRO DEGRADATION CHARACTERISTICS OF COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS

3.1.1 PHOSPHATE BUFFERED SALINE DEGRADATION

To assess the rate of degradation of the collagen based scaffolds (which had undergone prior crosslinking with both DHT and EDAC) by non-enzymatic mechanisms degradation was first measured in a phosphate buffered saline (PBS) degradation media.

In the PBS degradation media there was no significant degradation of either the pure collagen or CollGAG scaffolds. The CCP scaffolds however degraded approximately 30% during the first day of the experiment, a significant difference to the collagen and CollGAG scaffolds ($p=0.0674$), with little further degradation seen for the remaining 42 days (**Figure 3.1**).

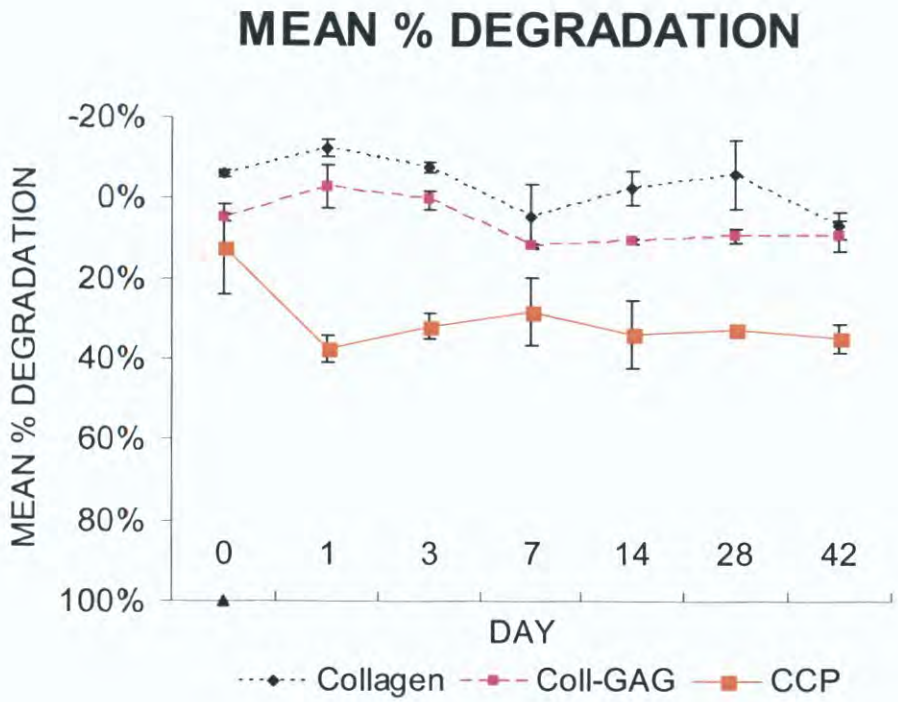

Figure 3.1 *Percentage scaffold degradation in phosphate buffered saline (PBS) over a 42 day period (n=4 for each scaffold material at each time point) (error bars correspond to standard deviation of the mean). Significant degradation occurred in the CCP scaffolds during the first day, as opposed to the collagen and Coll-GAG scaffolds (p=0.0674).*

Following manufacture, identically sized CCP scaffolds were significantly heavier than their collagen and Coll-GAG controls (Mean ± SD Dry weight: 17.75 ± 2.2 mg versus 1.95 ± 0.08 and 1.86 ± 0.09) ($p<0.0001$). Similar to the early change in degradation rate there was a corresponding initial loss of scaffold weight which subsequently plateaued after the 24 hour time point (**Figure 3.2**).

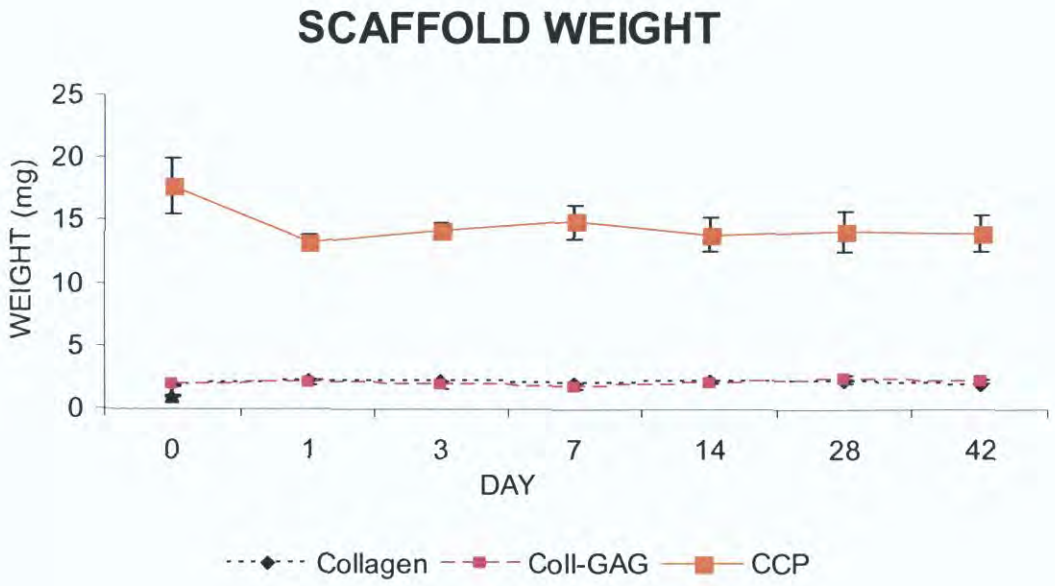

Figure 3.2 *The dry weight of scaffolds at each time point after exposure to the degradation media followed by freeze drying (n=4 at each time point) (error bars correspond to standard deviation of the mean).*

MECHANICAL TESTING

Wet compression mechanical testing was performed in PBS immediately after scaffold removal from the incubator revealing no significant change in scaffold mechanical properties over time in the PBS degradation media (Dunnett Contrast). The CCP scaffolds had significantly better mechanical properties compared to the collagen and Coll-GAG scaffolds at the beginning and end of the experiment ($P<0.0001$ & $p=0.0003$) (**Figure 3.3**).

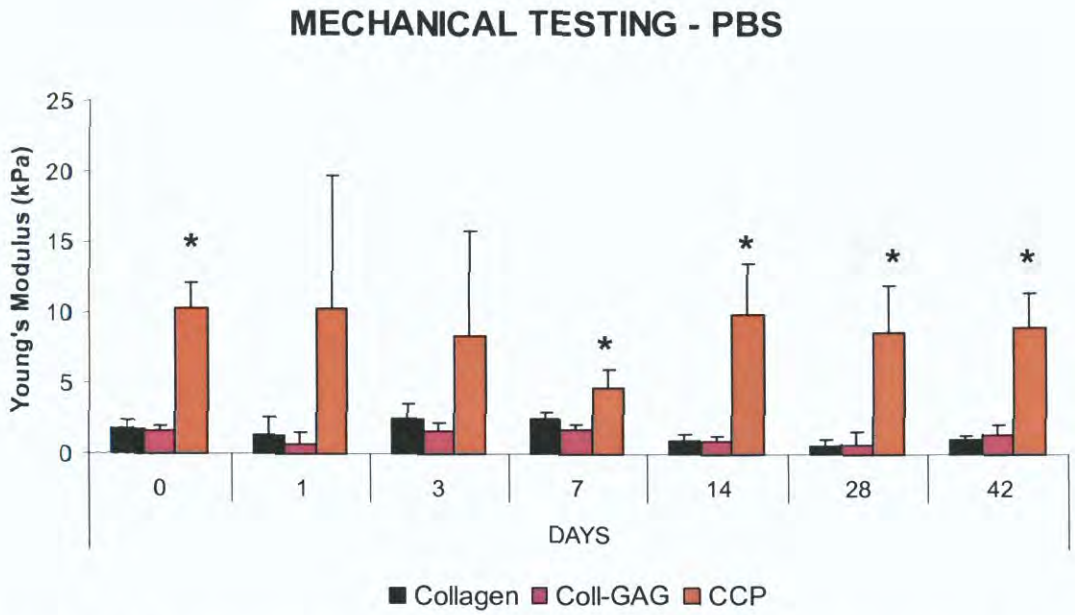

Figure 3.3 *Compressive modulus (a measure of mechanical stiffness) of each scaffold type at the various time points after incubation in PBS only (n=4 for each group) (error bars correspond to standard deviation of the mean). * p <0.05, by 1 way Anova. Significance values refer to differences between all scaffolds at each time point.*

IMAGING

Digital photographs of scaffolds at each time give a pictorial confirmation of the qualitative degradation results (**Figure 3.4**). There was no macroscopic evidence of degradation in either scaffold when incubated in the PBS degradation media.

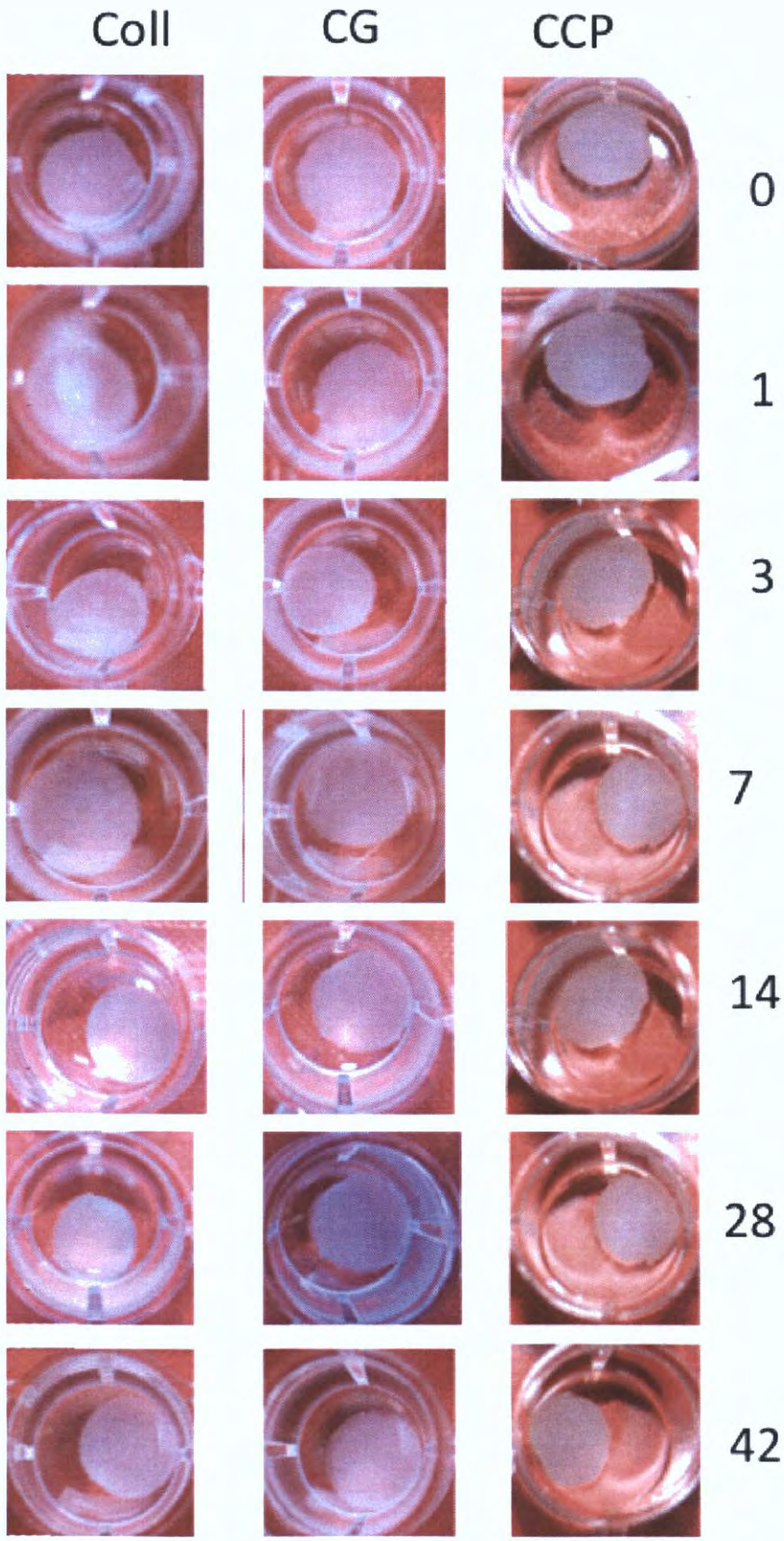

Figure 3.4 *Digital photographs of representative scaffolds at each time point (0 – 42 days) in the PBS degradation media. No scaffolds show macroscopic evidence of degradation.*

3.1.2 COLLAGENASE

DEGRADATION

Having seen only *limited* degradation in a PBS degradation media, the rate of degradation was then measured in a media containing collagenolytic enzymes.

In the bacterial collagenase media both the collagen and Coll-GAG scaffolds rapidly degraded after a critical time point (12 and 24 hours respectively). At the final time point of this experiment (72hrs) there was no significant degradation of the CCP scaffolds apart from that seen in the first hour of incubation (**Figure 3.4**).

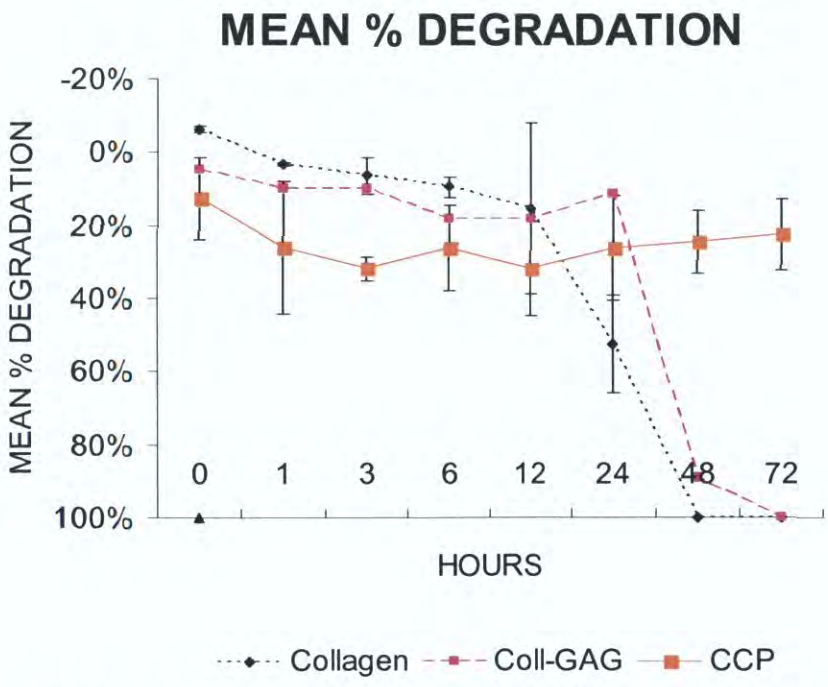

***Figure 3.5**. Percentage scaffold degradation in 0.05 mg/ml collagenase over a 72 hour time period (n=4 for each scaffold material at each time point) (error bars correspond to standard deviation of the mean).*

Despite the initial rate of degradation, the CCP scaffolds were significantly heavier than the collagen and Coll-GAG controls at all time points ($p<0.0001$) (**Figure 3.5**)

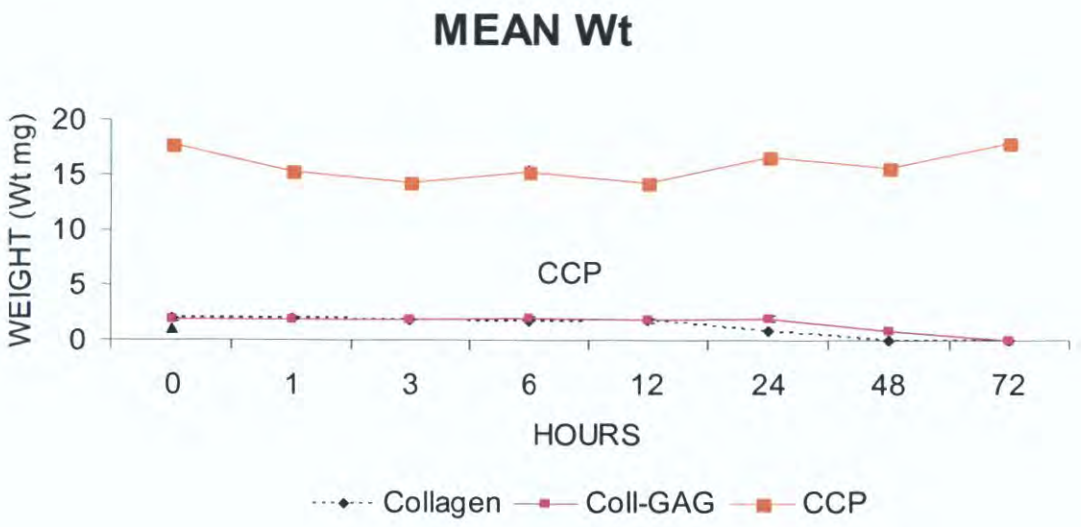

Figure 3.6 *The dry weight of scaffolds at each time point after exposure to 0.05 mg/ml collagenase degradation media followed by freeze drying (n=4 at each time point) (error bars correspond to standard deviation of the mean).*

MECHANICAL TESTING

Mechanical testing of scaffolds following removal from the collagenase degradation media revealed a progressive loss of mechanical properties over time for all scaffold groups (**Figure 3.6**). Collagen and Coll-GAG scaffolds showed no significant change in mechanical properties up to 12 hrs (when scaffold degradation began). The CCP had a significant loss in mechanical properties after 12hrs of incubation compared to the mechanical properties at 0hrs, despite only limited degradation of the scaffold being evident ($p=0.0096$).

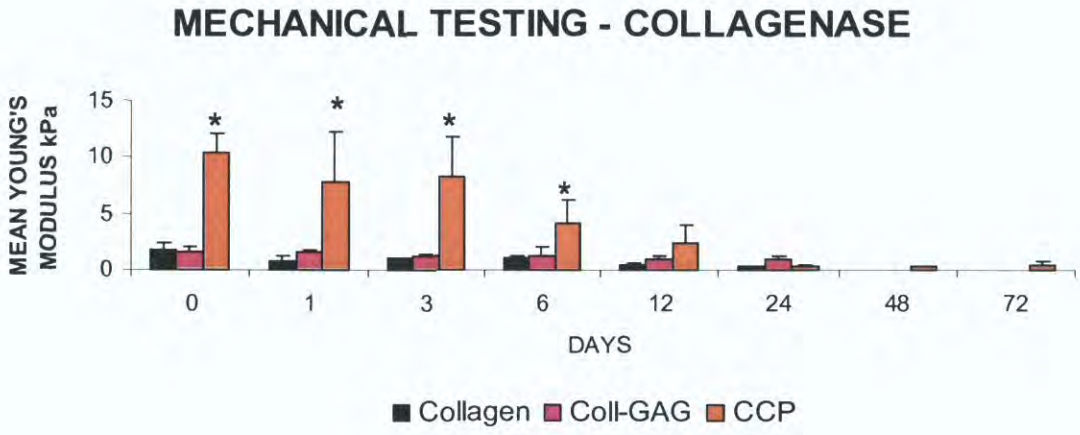

Figure 3.7 *Compressive modulus (a measure of mechanical stiffness) of each scaffold type at the various time points after incubation in collagenase (n=4 for each group) (error bars correspond to standard deviation of the mean).). * p <0.05, by 1 way Anova. Significance values refer to differences between all scaffolds at each time point.*

IMAGING

Digital photographs of scaffolds at each time give a pictorial confirmation of the qualitative degradation results (**Figure 3.8**). In the collagenase degradation media complete degradation is seen at 48hrs for the collagen scaffolds, and almost complete degradation of the Coll-GAG scaffolds. CCP scaffolds are however macroscopically intact at 72hrs.

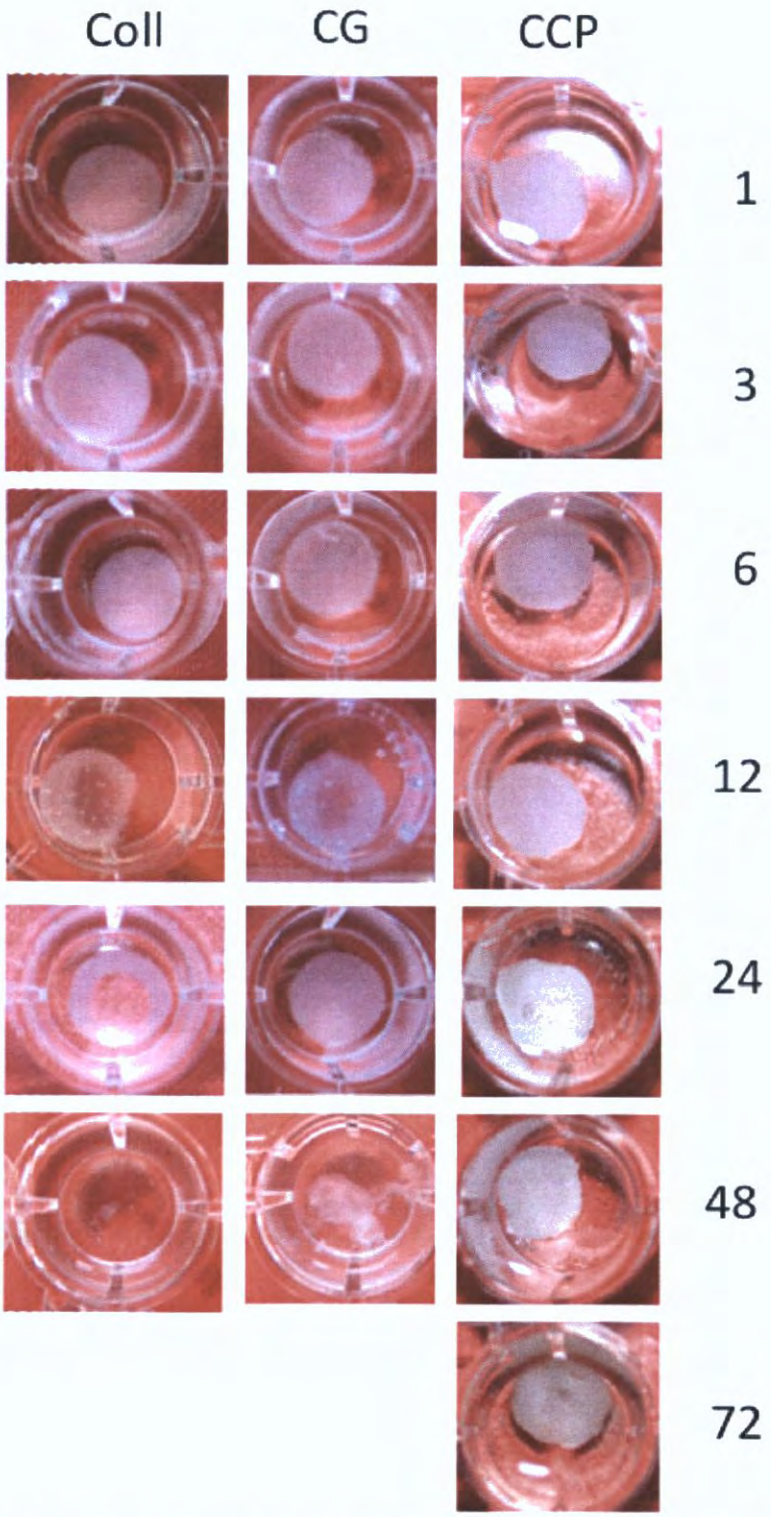

Figure 3.8 *Digital photographs of representative scaffolds at each time point (1- 72 hrs) in the collagenase degradation media. The collagen scaffolds were fully degraded at 48hrs, the collagen GAG scaffolds were almost completely degraded at 48hrs, whilst the collagen CP scaffolds showed only minimal macroscopic degradation at 72 hrs.*

3.2 IN VIVO ASSESMENT OF SCAFFOLD DEGRADATION CHARACTERISTICS AND HOST IMMUNE RESPONSE TO IMPLANTED COLLAGEN AND MINERALISED COLLAGEN SCAFFOLDS WITH AND WITHOUT MESENCYMAL STEM CELL SEEDING

3.2.1 HISTOLOGY

Histological analysis was performed on H&E prepared specimen to assess the outcomes of the scaffolds and their success in new bone formation having been implanted into the rat calvaria.

Qualitative assessment of H&E stained slides revealed minimal bone healing in the empty defect group (**Figure 3.9**). In those specimens where a collagen scaffold had been implanted there was variable evidence of new bone formation.

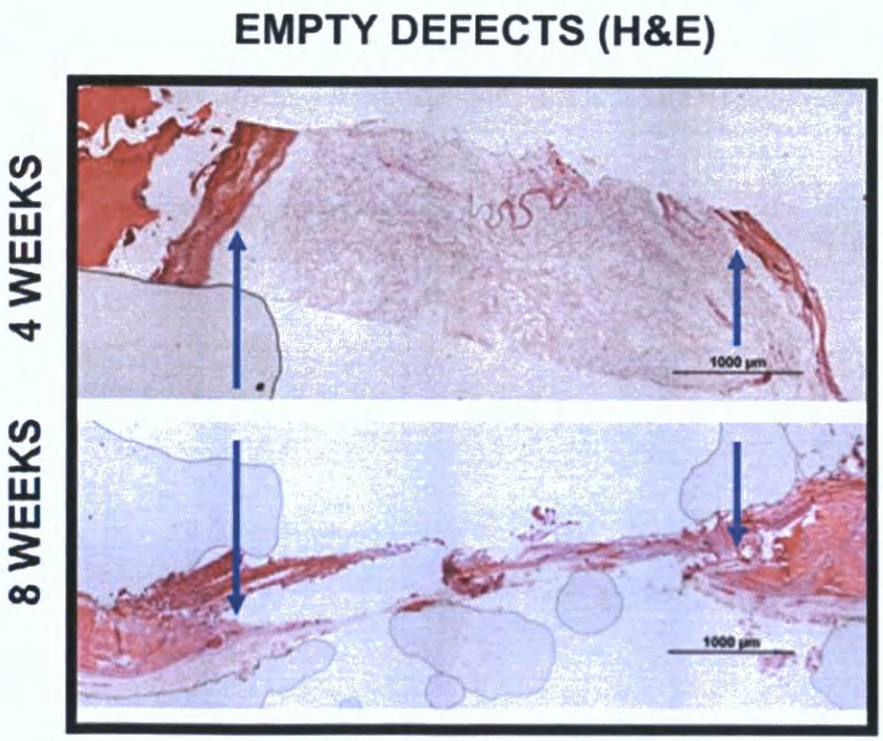

Figure 3.9 *Merged low powered magnification (x4) of H&E stained empty defect control specimens at 4 and 8 weeks, post defect creation. The edges of the critically sized defect are labelled with blue arrows, with the defect filled with fibrous tissue.*

In the non cell seeded CCP scaffolds (**Figure 3.10**) there were early signs of mineralisation and new bone formation at 4 weeks, which was associated with inflammatory cells aggregated at the interface of host and new bone. At 8 weeks there was diffuse new bone formation both bridging the defect and within the substance of the scaffold. The inflammatory reaction had subsided apart from at isolated areas of scaffold which remained unmineralised. Neovascularisation was evident at 4 weeks and pronounced at 8 weeks. Those CCP scaffolds which were MSC pre-seeded demonstrated poor new bone formation or mineralisation (examined using polarised light) with an intense inflammatory reaction around the periphery of the scaffold. This dense collagen based fibrous "capsule" was heavily laden with fibroblasts and inflammatory cells including some giant cells. The remaining inner scaffold was largely empty and devoid of vascularisation.

The non cell seeded CollGAG scaffolds showed areas of mineralisation at 4 and 8 weeks with evidence of inflammatory cells and the occasional giant cell. The extent of new bone formation / mineralisation was appreciably less than in the corresponding non-cell seeded CCP scaffolds. At 4 weeks the cell seeded CollGAG scaffolds demonstrated a mild inflammatory reaction similar but less severe to the "capsule" surrounding the cell seeded collagen CP scaffolds. At this time there were very small areas of new bone formation and mineralisation visible with polarized light at the periphery of the scaffold/defect. At 8 weeks this inflammatory response had largely abated with areas of new bone formation evident both at the edge of the host bone and the occasional island of mineralisation within the scaffold (**Figure 3.11**).

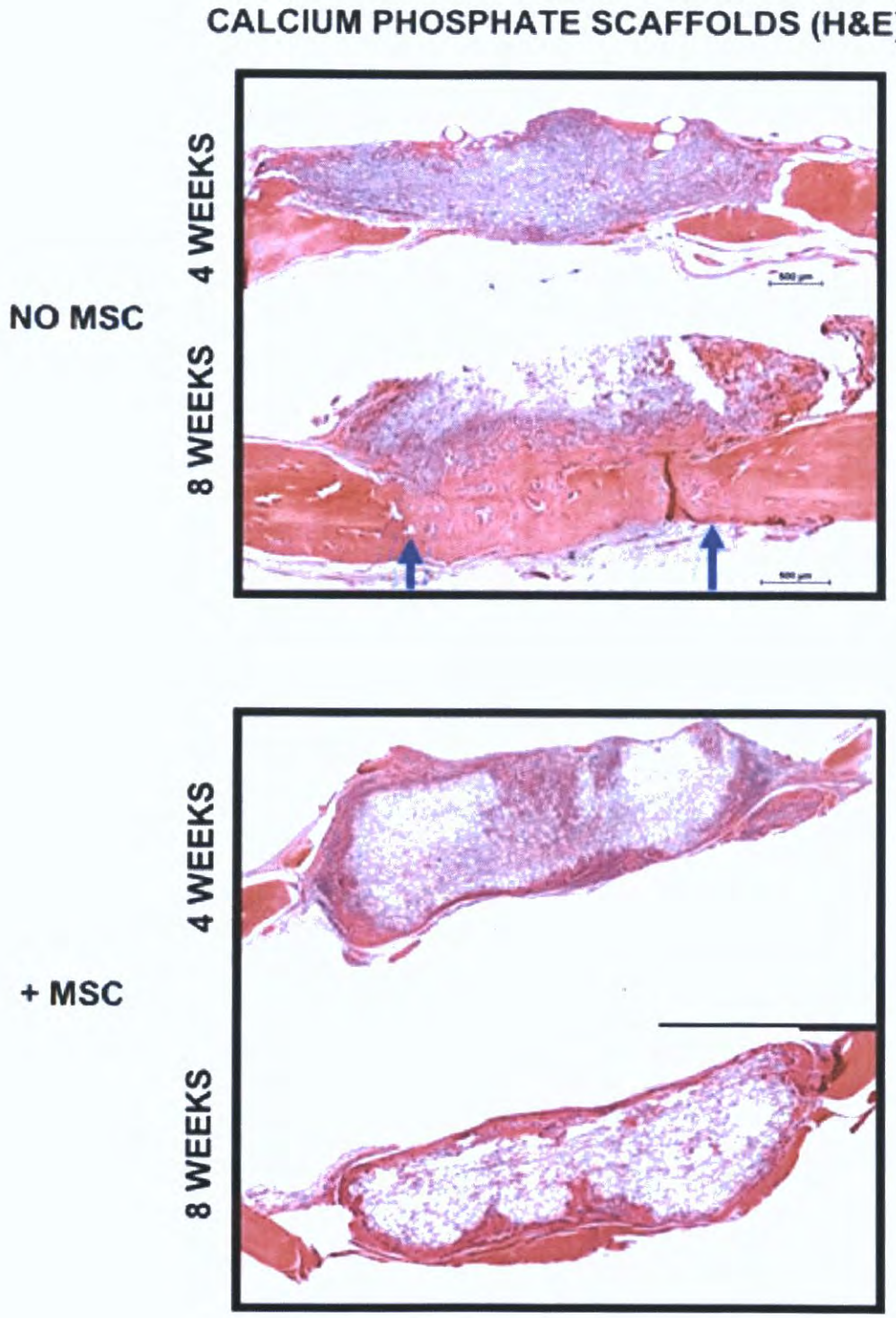

Figure 3.10 *Merged low powered magnification (x4) of H&E stained specimens implanted with CCP scaffolds at 4 and 8 weeks, with and without the prior culture of mesenchymal stem cells. New bone formation was most abundant in non-cell seeded scaffolds at 8 weeks where the defect is bridged by new bone (blue arrows). Cell seeded scaffolds were associated with a dense inflammatory reaction surrounding the periphery of the scaffold at both 4 and 8 weeks. Scaffold degradation is obvious at 8 weeks in the non-cell*

seeded scaffolds, whereas little scaffold degradation was seen at either time point in the cell seeded scaffolds.

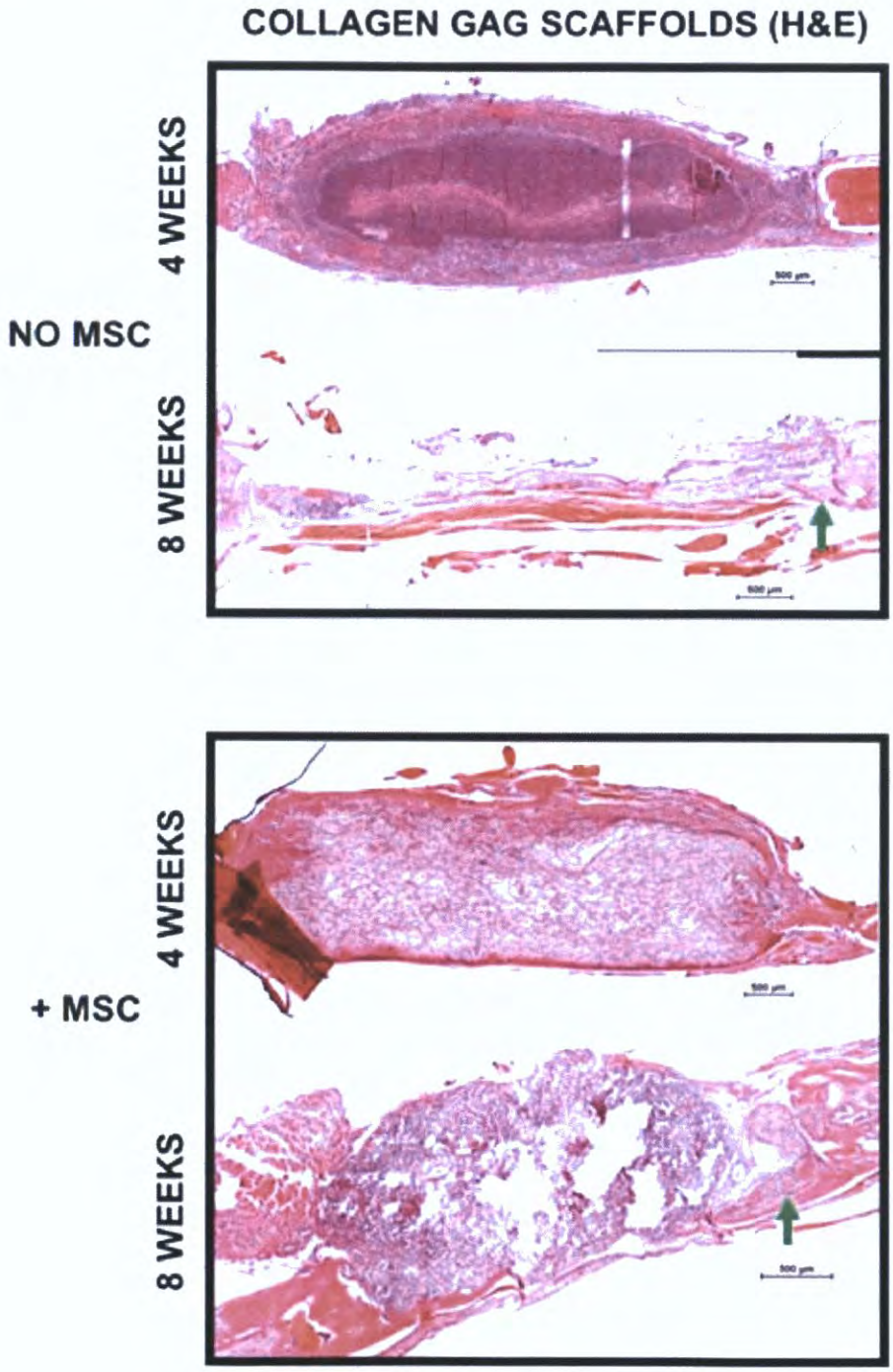

Figure 3.11 *Merged low powered magnification (x4) of H&E stained specimens implanted with CollGAG scaffolds at 4 and 8 weeks, with and without the prior application of mesenchymal stem cells. There was marked degradation of the non-cell seeded scaffolds at 8 weeks with small areas of new bone formation. Cell-seeded scaffolds were again associated with a peripheral inflammatory reaction at 4 weeks which had predominantly*

subsided at 8 weeks. New bone formation was more evident in both the non-cell seeded scaffolds and cell seeded scaffolds at 8 weeks (examples identified with green arrows), however to a lesser extent than seen in the non-cell seeded CCP scaffolds (Fig 3.10)

3.2.2 HISTOMORPHOMETRY

Qualitative analysis of the H&E sections revealed significantly higher rates of new bone formation in the non cell seed scaffolds (CCP and CollGAG) at both 4 and 8 weeks compared to both the cell seeded counterparts and the empty controls (**Figure 3.12**).

Figure 3.12a – CCP SCAFFOLDS

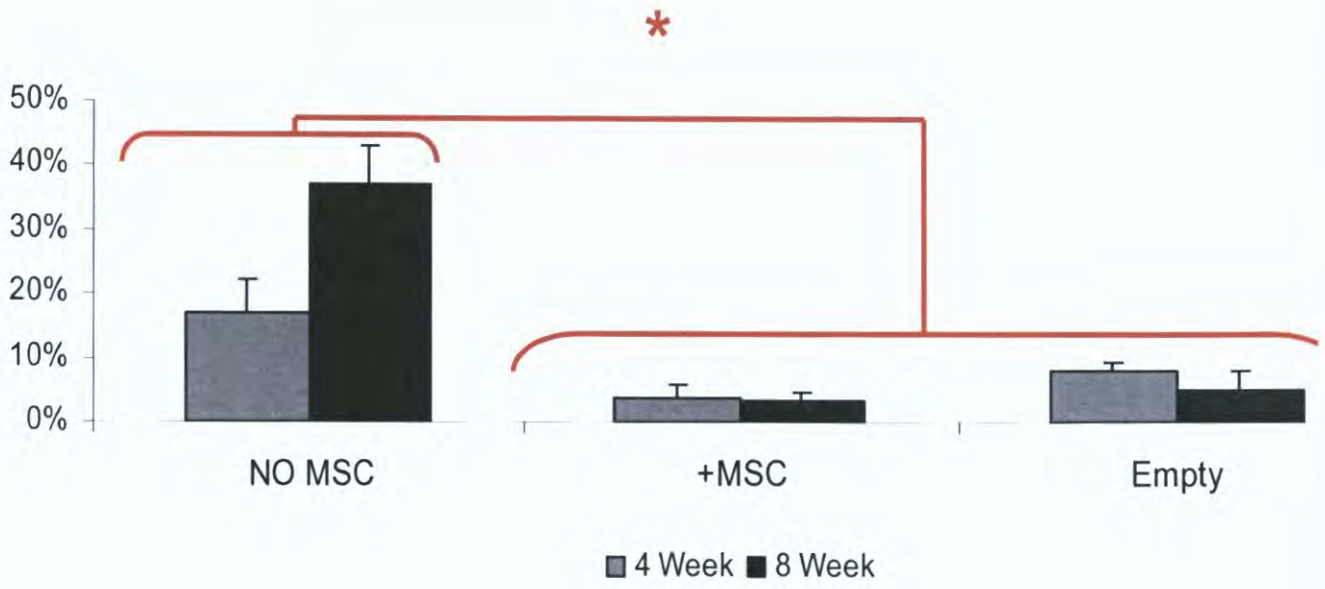

Figure 3.12b – CollGAG SCAFFOLDS

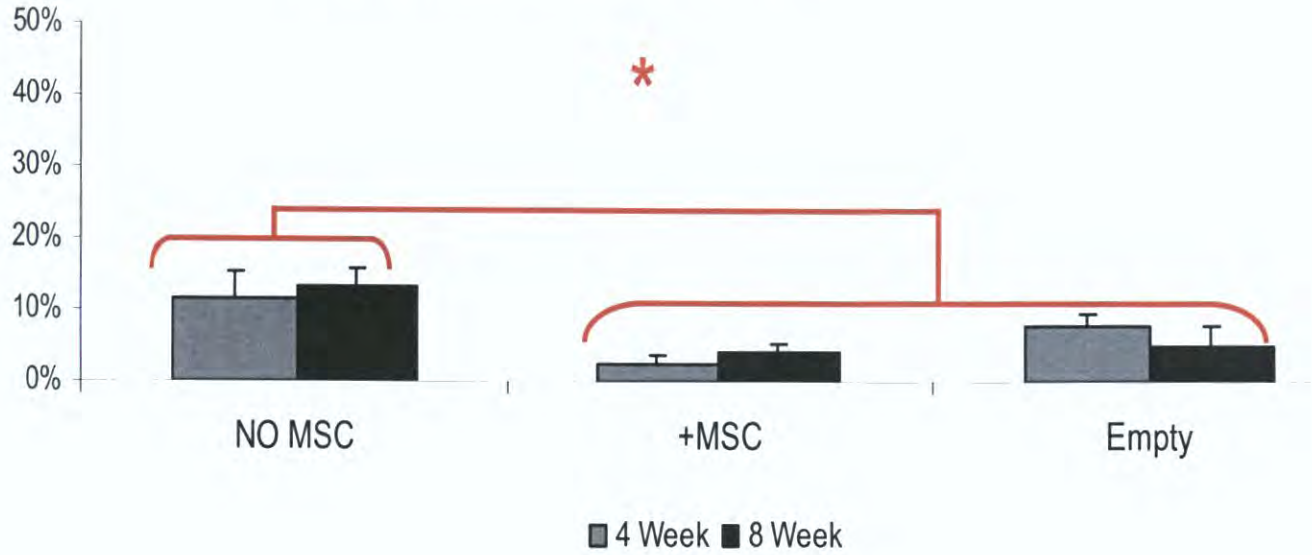

Figure 3.12 *Percentage of total defect area replaced by new bone formation as measured by computer histomorphometry in the CCP scaffolds (a) and CollGAG scaffolds (b). Comparison between groups was performed using 1-way anova with Tukey error protection. P<0.0001. Error bars correspond to standard deviation of the mean.*

The highest rate of new bone formation was found in the non-cell seeded CCP scaffolds with 17.05 ± 5.01% (Mean ± SD) at 4 weeks and 37.24 ± 5.75% at 8 weeks, as compared to 11.65 ± 3.87% (4 weeks) and 13.15 ± 2.59% (8 weeks) for the non-cell seeded CollGAG scaffolds and 7.92 ± 1.48 % (4 weeks) and 5.10 ± 2.82% (8 weeks) for the empty defect controls (**Figure 3.13**).

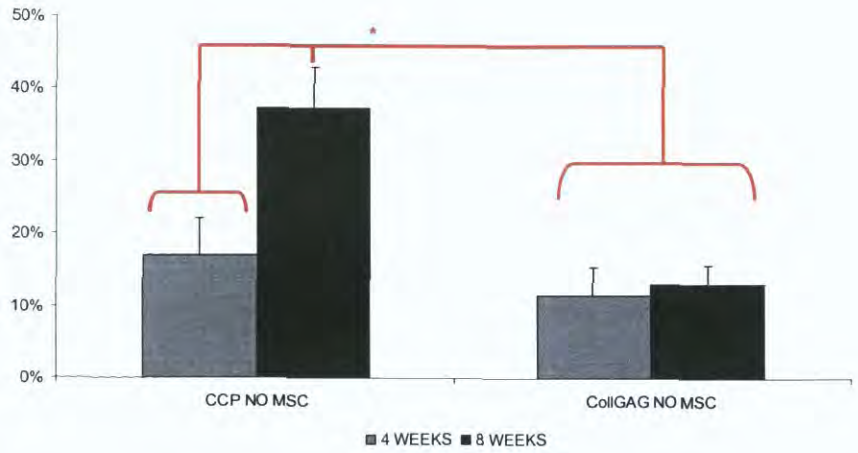

Figure 3.13 *Percentage of total defect area replaced by new bone formation as measured by computer histomorphometry in the non cell seeded CCP and Coll GAG scaffolds. The percentage new bone formation was significantly higher in CCP scaffolds at 8 weeks compared to CCP at 4 weeks and CollGAG scaffolds at both time points ($p<0.0001$). Error bars correspond to standard deviation of the mean.*

Qualitative assessment using histomorphometry was then performed to measure the area of remaining scaffold as an in-vivo measure of scaffold degradation. Scaffold degradation was significantly higher in the non cell seeded CollGAG scaffolds (0.98 ± 0.05 mm^2 (4 weeks), 0.32 ± 0.12 mm^2 (8 weeks)) than cell seeded scaffolds (5.50 ± 0.91 mm^2 (4 weeks), 5.66 ± 2.28 mm^2 (8 weeks)). There was no statistical difference identified between the amount of scaffold degradation in the two scaffold types at each time point; CCP non-cell seeded [5.69 ± 1.56 mm^2 (4 weeks), 2.88 ± 0.66 mm^2 (8 weeks)) or cell seeded scaffolds 5.01 ± 1.15 mm^2 (4 weeks), 4.26 ± 2.07 mm^2 (8 weeks) $p=0.5806$ (1-Way Anova)]. However in the CCP scaffolds there was a significant difference between the amount of scaffolds remaining at 4 and 8 weeks in the non-cell seeded scaffolds, $p=0.0210$ (Independent t-test) (**Figure 3.14**).

Figure 3.14a – CCP SCAFFOLDS

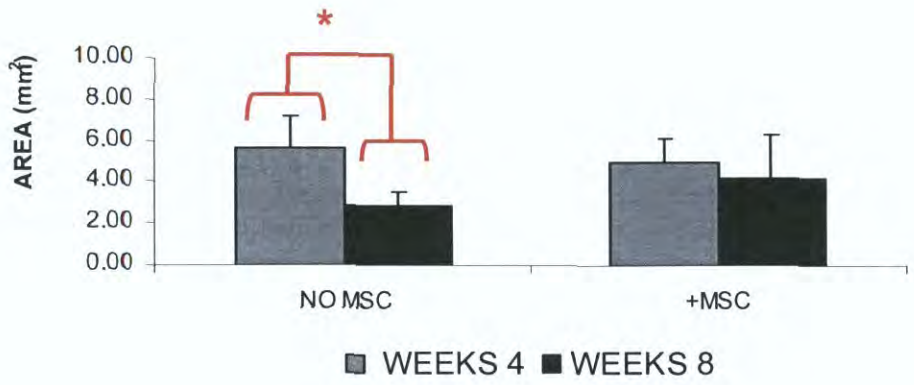

Figure 3.14b – CollGAG SCAFFOLDS

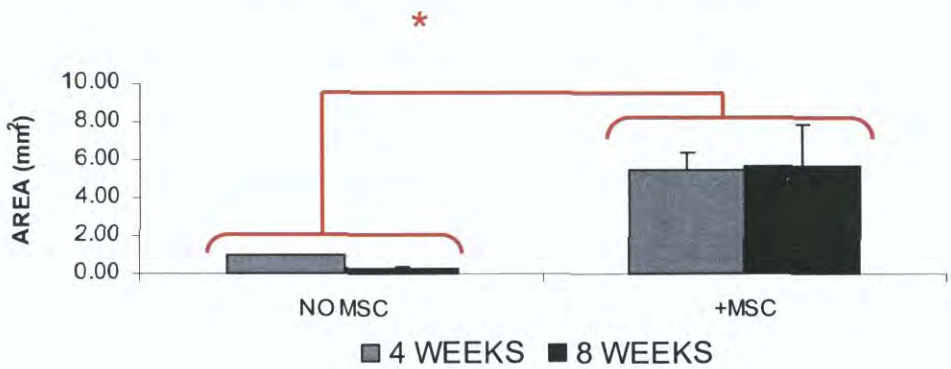

Figure 3.14 *The amount of in vivo scaffold degradation as measured by the area of remaining scaffold using histomorphometry in the CCP scaffolds (a) and CollGAG scaffolds (b). Comparison between groups was performed using 1-way anova with Tukey error protection. P<0.05. Error bars correspond to the standard deviation of the mean.*

3.2.3 MACROPHAGE PHENOTYPE

In view of the knowledge that macrophages play a role in initial inflammatory phase of fracture healing and that we had demonstrated an inflammatory response to some of our scaffolds, immunohistochemical staining was performed to determine the presence of macrophages. Further analysis was then performed to determine the phenotype of any macrophage response, as macrophage activity has recently been phenotyped as either pro-inflammatory (M1) or pro-wound healing (Badylak, Valentin et al. 2008).

CCP SCAFFOLDS

Staining for the pan macrophage marker CD68 demonstrated the presence of macrophages in the capsule at the periphery of MSC seeded CCP scaffolds, but little macrophage activity in non-cell seeded scaffolds. This macrophage response was more pronounced at 4 than 8 weeks (**Figure 3.15**). Staining for the immunomodulatory and tissue remodelling (M2 phenotype) marker CD 163 at 4 weeks demonstrated active M2 mononuclear cell activity in the scaffold substance, particularly at sites of new bone formation and the host – scaffold interface in non MSC seeded scaffolds. These mononuclear cells were not as evident at 8 weeks. In the cell seeded scaffolds, M2 phenotype mononuclear cells were evident at both 4 and 8 weeks predominantly at the periphery of the scaffold, especially in the fibrous / inflammatory capsule seen previously on histological examination (**Figure 3.16**).

The pro-inflammatory (M1 phenotype) marker CCR7 staining demonstrated little M1 phenotype mononuclear cell activity in non-cell seeded scaffolds at

either 4 or 8 weeks but a marked population of such inflammatory cells at the periphery of cell seeded scaffolds at both 4 and 8 weeks (**Figure 3.17**).

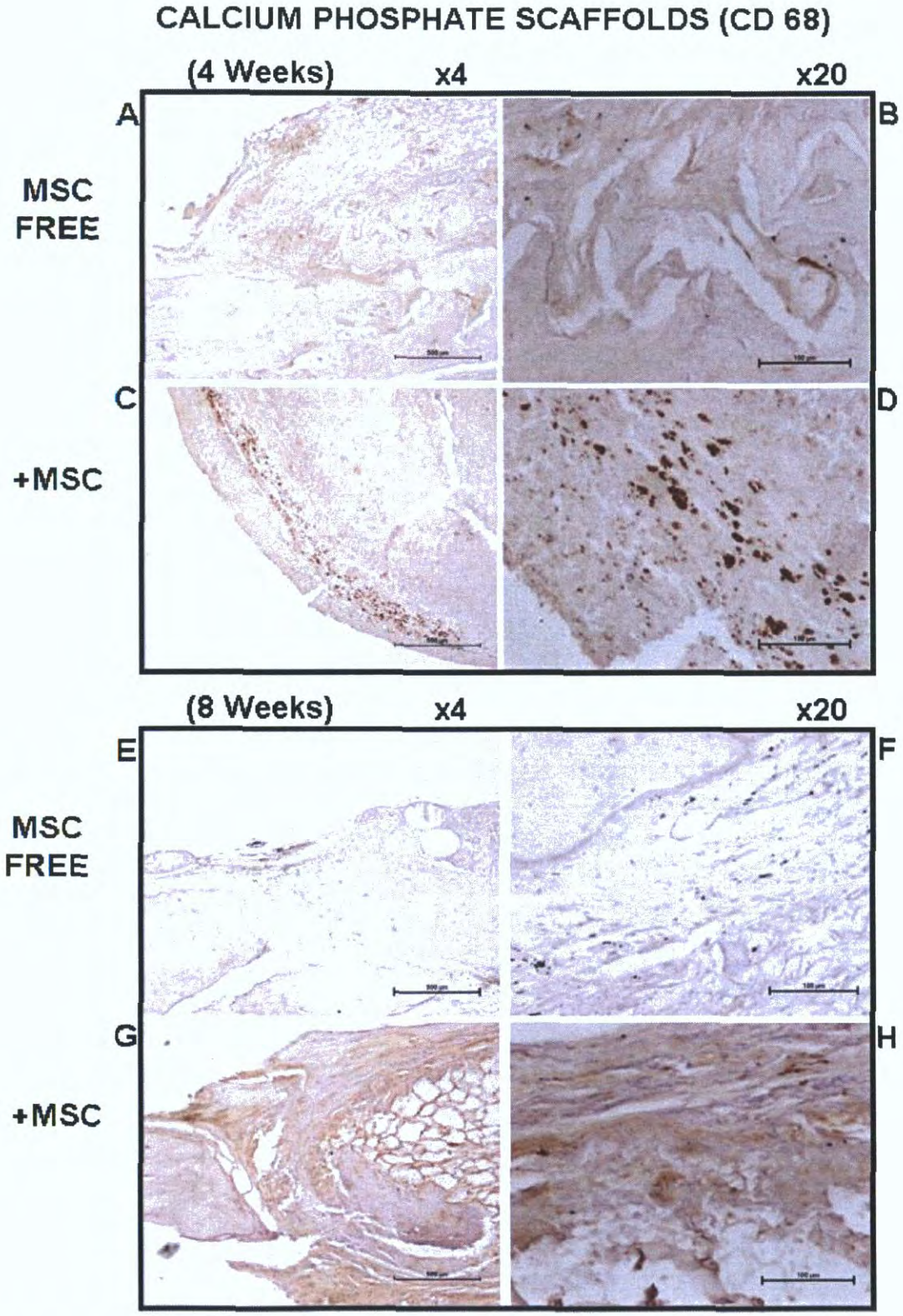

Figure 3.15 *CD68 (Pan-macrophage) immunostaining of collagen CP scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks. Specimens pre-cultured with MSCs(C,D,G,H) showed marked positive staining (brown) at the scaffold periphery.*

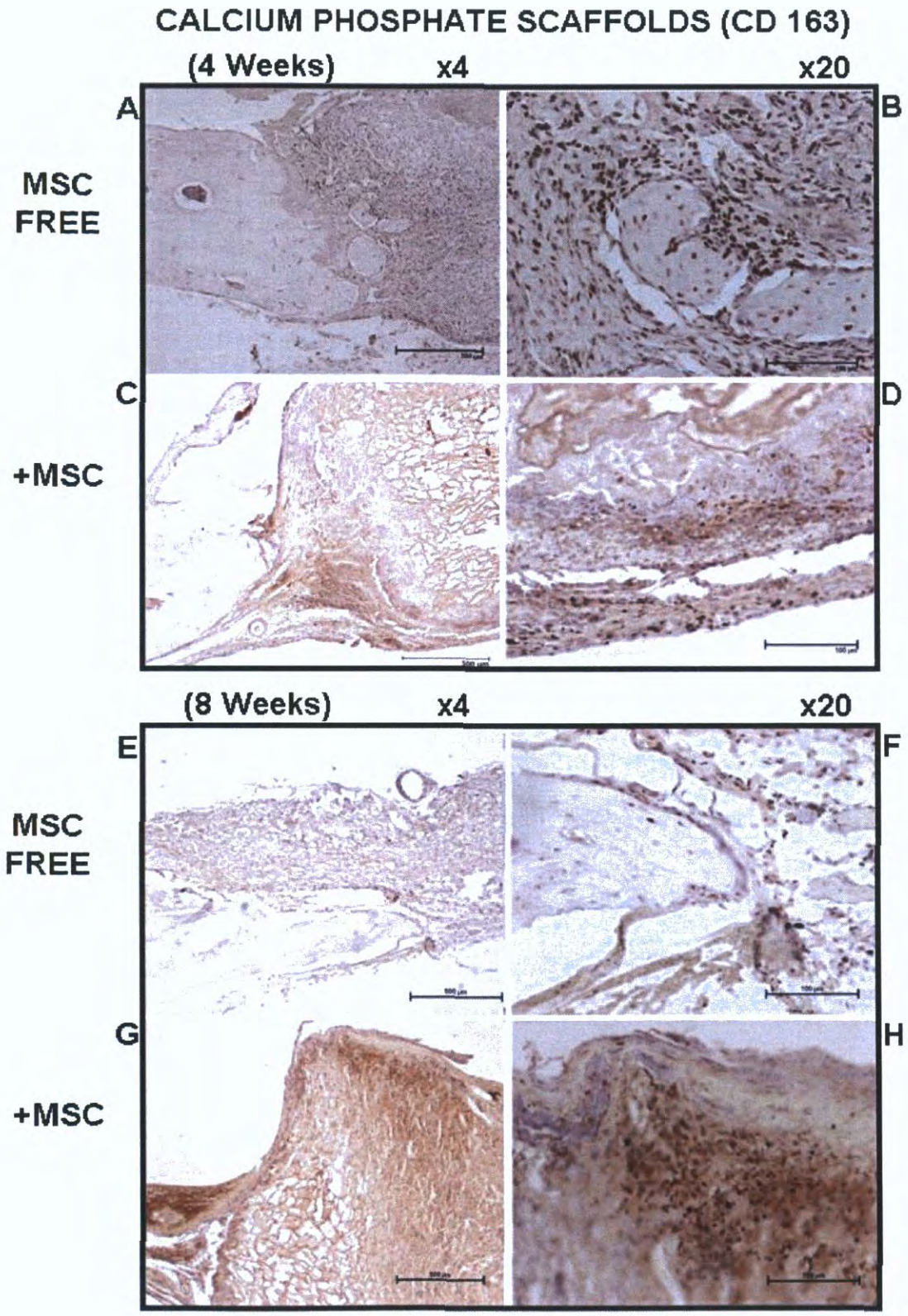

Figure 3.16 *CD163 (M2 Phenotype) immunostaining of collagen CP scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks. M2 macrophage activity is seen at the periphery of MSC pre-seeded scaffolds (C,D,G,H) but at sites of new bone formation in non MSC cultured scaffolds (A,B,E,F).*

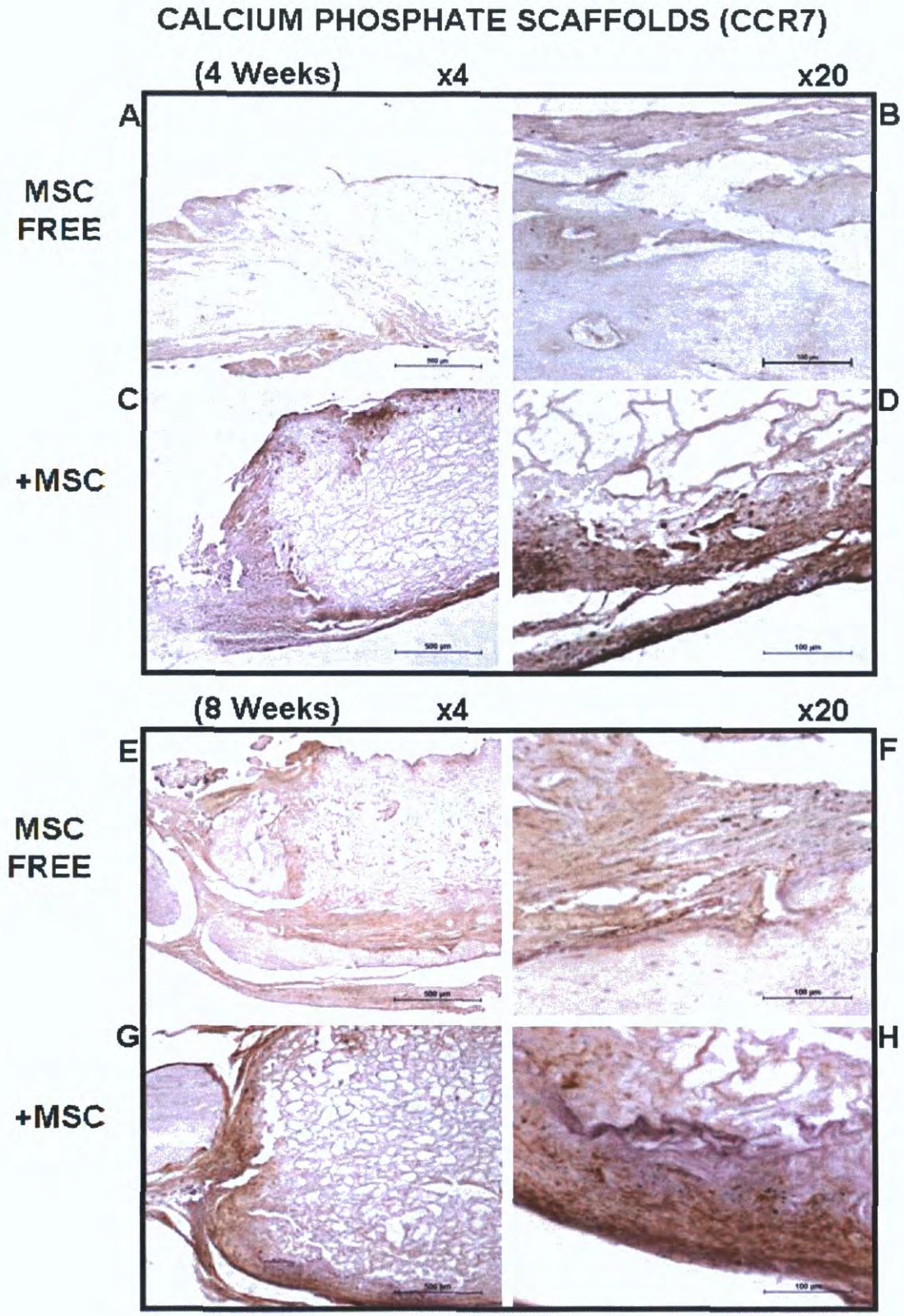

Figure 3.17 *CCR7 (M1 Phenotype) immunostaining of collagen CP scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks. Marked M1 macrophage activity was seen at the periphery of MSC seeded scaffolds (C,D,G,H).*

COLLAGEN GAG SCAFFOLDS

In the collagen GAG scaffolds there was only minimal staining for the pan-macrophage marker CD68 in non-cell seeded scaffolds but abundant activity at the periphery of scaffolds which were previously cell seeded, at both 4 and 8 weeks (**Figure 3.18** – collagen GAG CD68).

In these CollGAG scaffolds which had less new bone formation than the CCP scaffolds, M2 phenotype mononuclear cells were predominantly evident at the host – scaffold interface at 4 weeks with some cells emerging in the scaffold itself at 8 weeks as islands of new bone became evident. In the cell-seeded samples these mononuclear cells were only present at the periphery of the scaffold, with little if any activity at the host-scaffold interface or inside the substance of the scaffold (**Figure 3.19** – collagen GAG CD163).

Pro-inflammatory M1 mononuclear cells were present at the periphery of non-cell seeded scaffolds at 4 weeks but were not evident at 8 weeks. In the cell seeded collagen GAG scaffolds, these pro-inflammatory phenotype macrophages were widely evident at the periphery and extending some way into the substance of scaffolds at both 4 and 8 weeks. (**Figure 3.20** – collagen GAG CCR7)

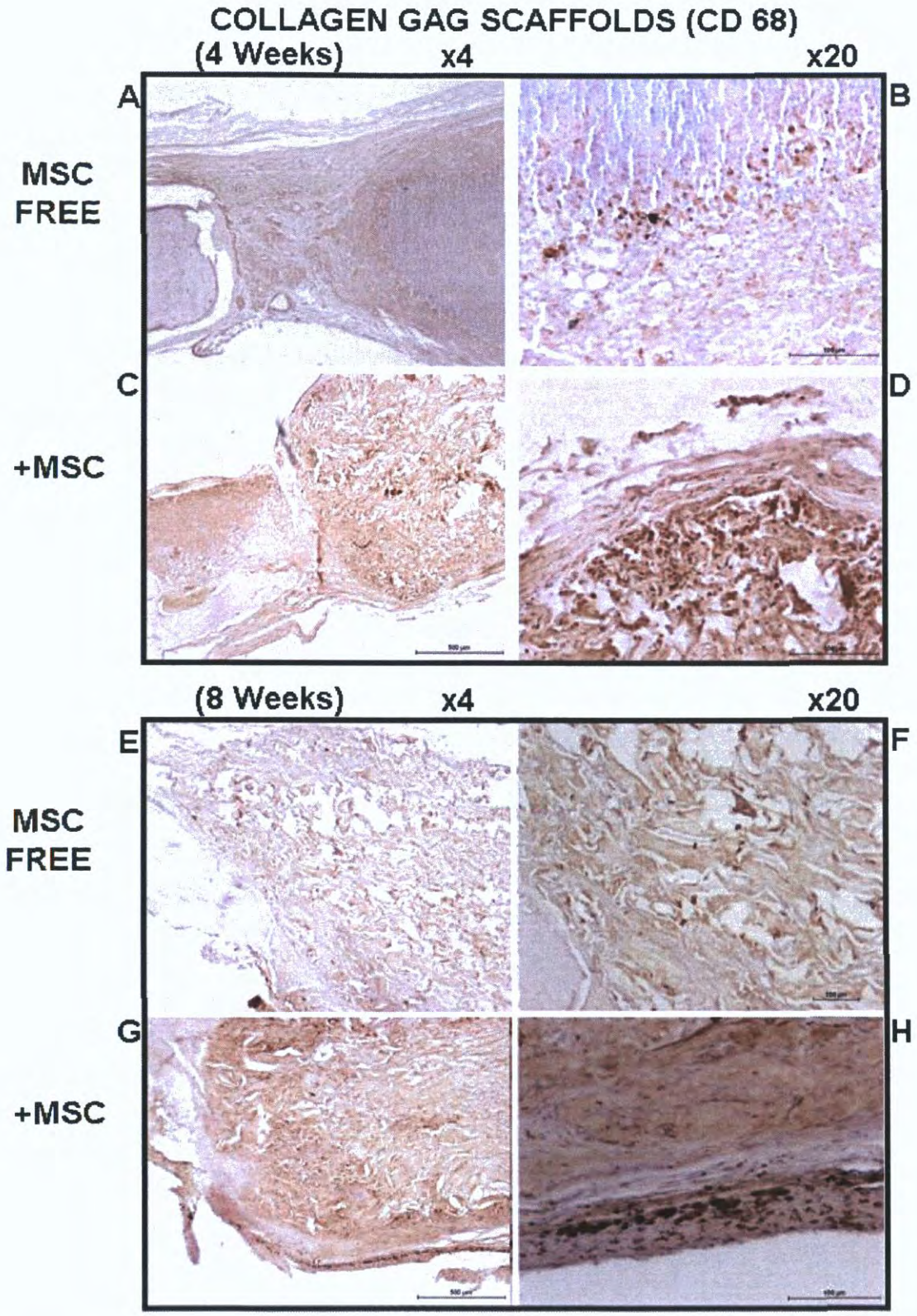

Figure 3.18 *CD68 (Panmacrophage) immunostaining of collagen GAG scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks.*

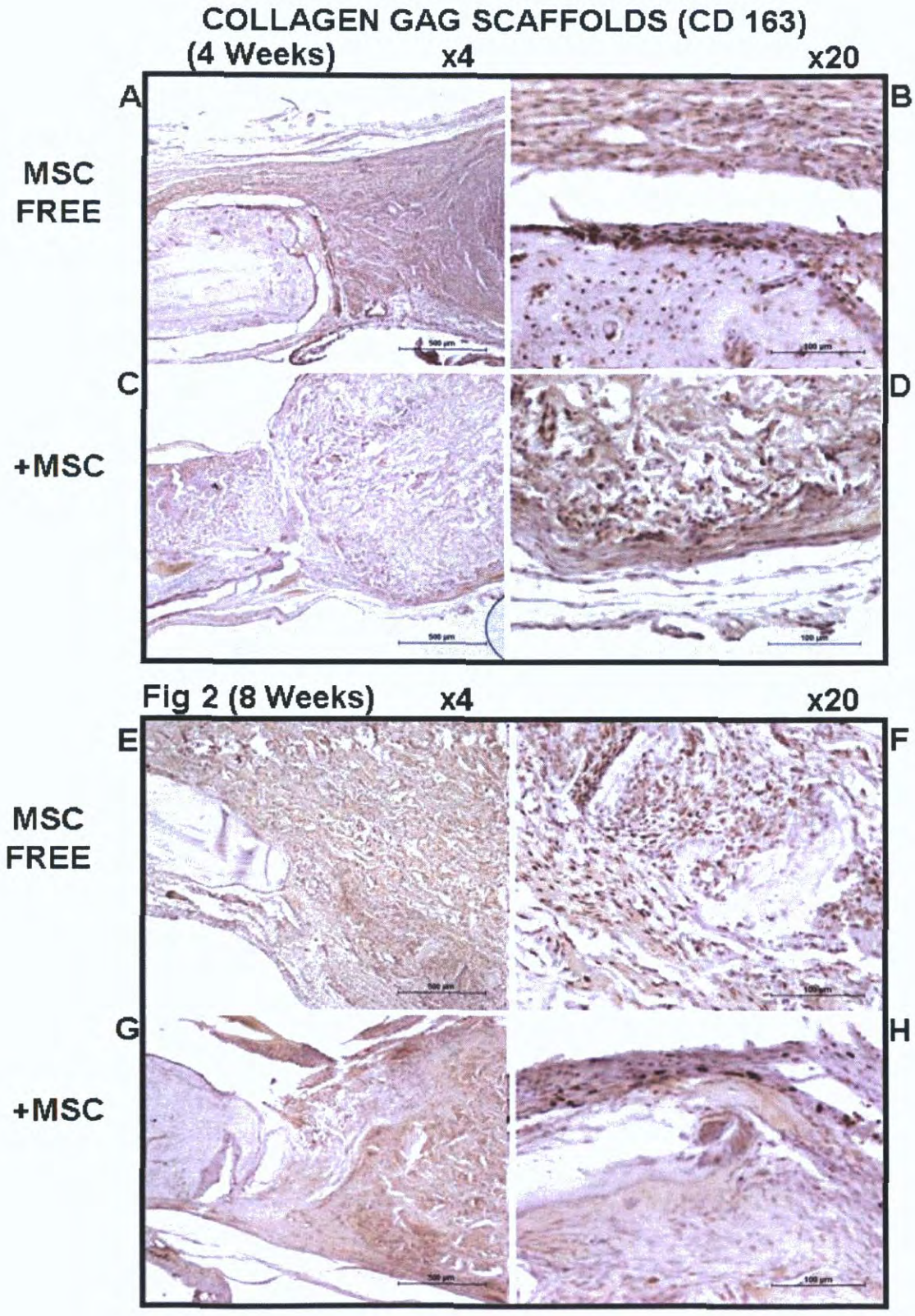

Figure 3.19 *CD163 (M2 Phenotype) immunostaining of collagen GAG scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks.*

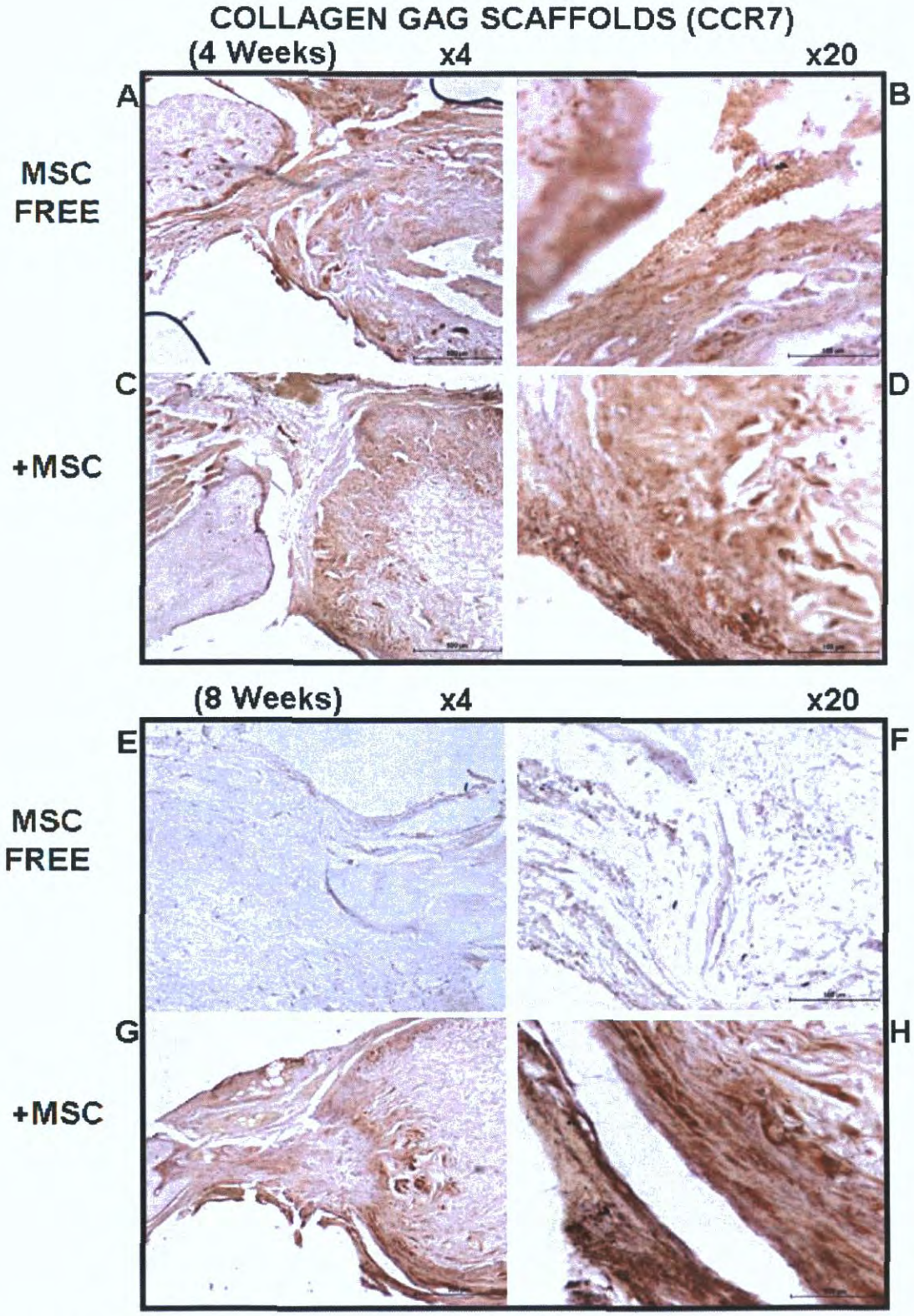

Figure 3.20 *CCR7 (M1 Phenotype) immunostaining of collagen GAG scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks. Staining was widely evident at 4 in all scaffolds (A-D), but had subsided in non MSC seeded scaffolds at 8 weeks (E,F) but not in MSC seeded scaffolds (G,H).*

3.2.4 COLLAGENASE 3 / MMP-13

MMP 13 is a collagenolytic known to play a role in fracture healing and bone remodelling (Kosaki, Takaishi et al. 2007). In this knowledge we performed Immunohistochemical staining for this enzyme to determine it's role in collagen based scaffold degradation and new bone formation.

Staining for MMP-13 (Collagenase type 3) was evident at both time points in the empty defect specimens (**Figure 3.21**). Diffuse staining was seen throughout the fibrous tissue aggregated in the calvarial defect whilst intense staining was seen at a probable osteoclastic resorption zone (**Figure 3.21 B**). A similar pattern was noted in specimens where scaffolds had been implanted into the defect. Both CCP and ColIGAG scaffolds were characterised by diffuse background staining of the scaffold with marked staining seen at the host bone / scaffold interface (**Figure 3.22 and 3.23**). Avid staining was seen at sites of intense new bone formation in CCP scaffolds at 4 weeks (**Figure 3.22B**).

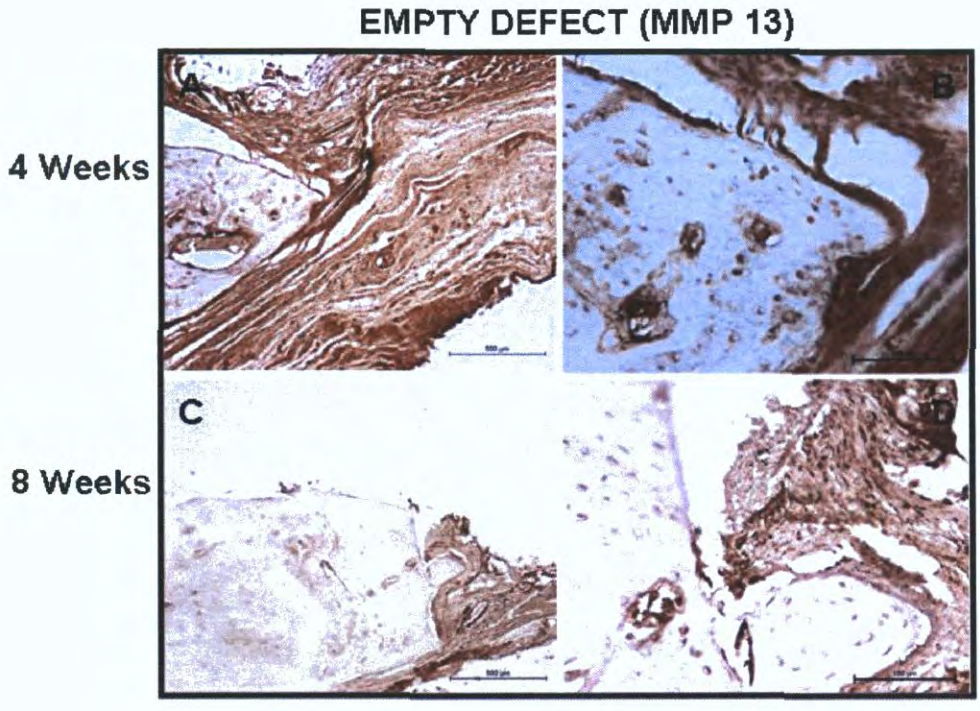

Figure 3.21 *MMP13 immunostaining of empty defects at both low (x4) and high power (x20) at 4 and 8 weeks.*

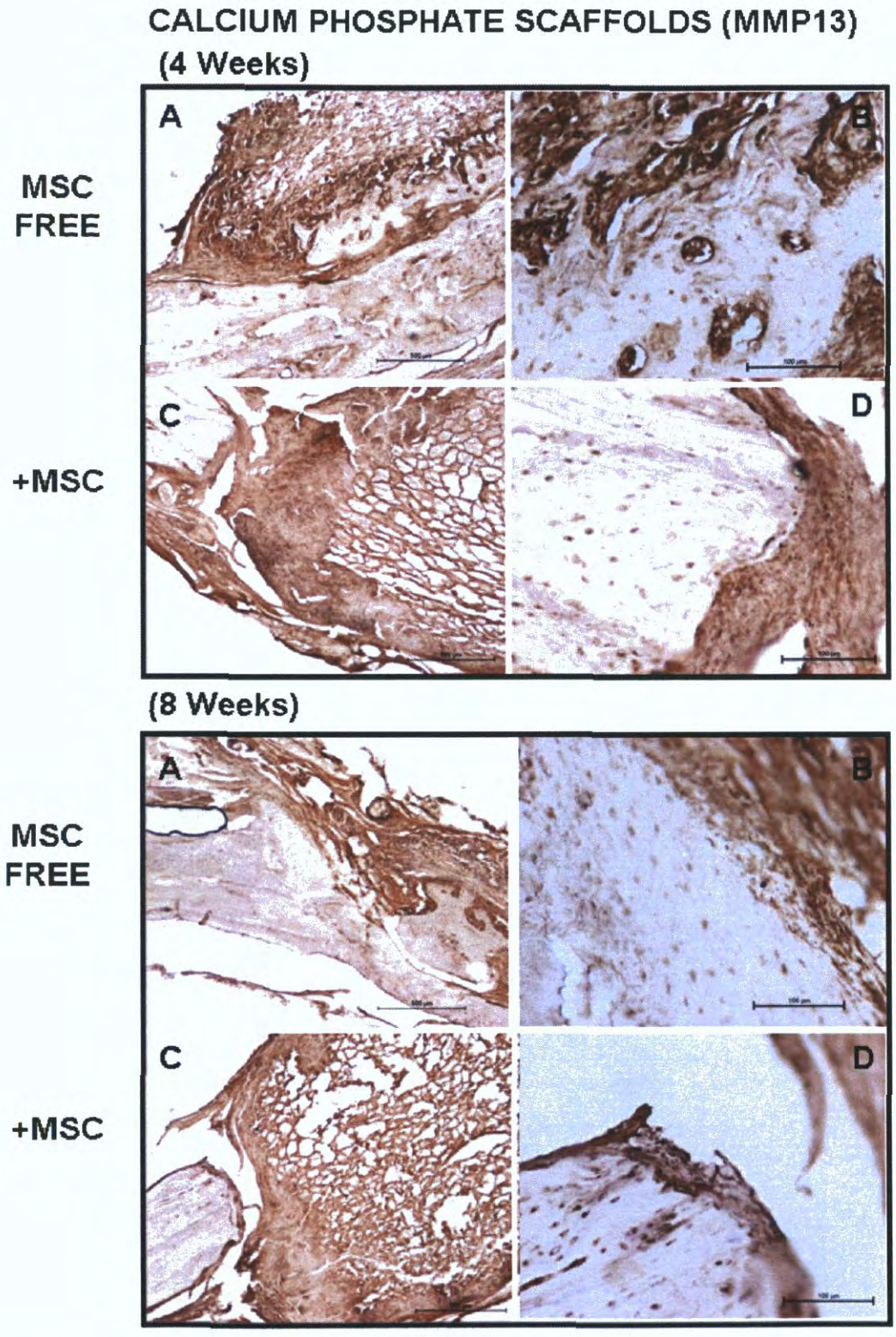

Figure 3.22 *MMP13 immunostaining of collagen CP scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks.*

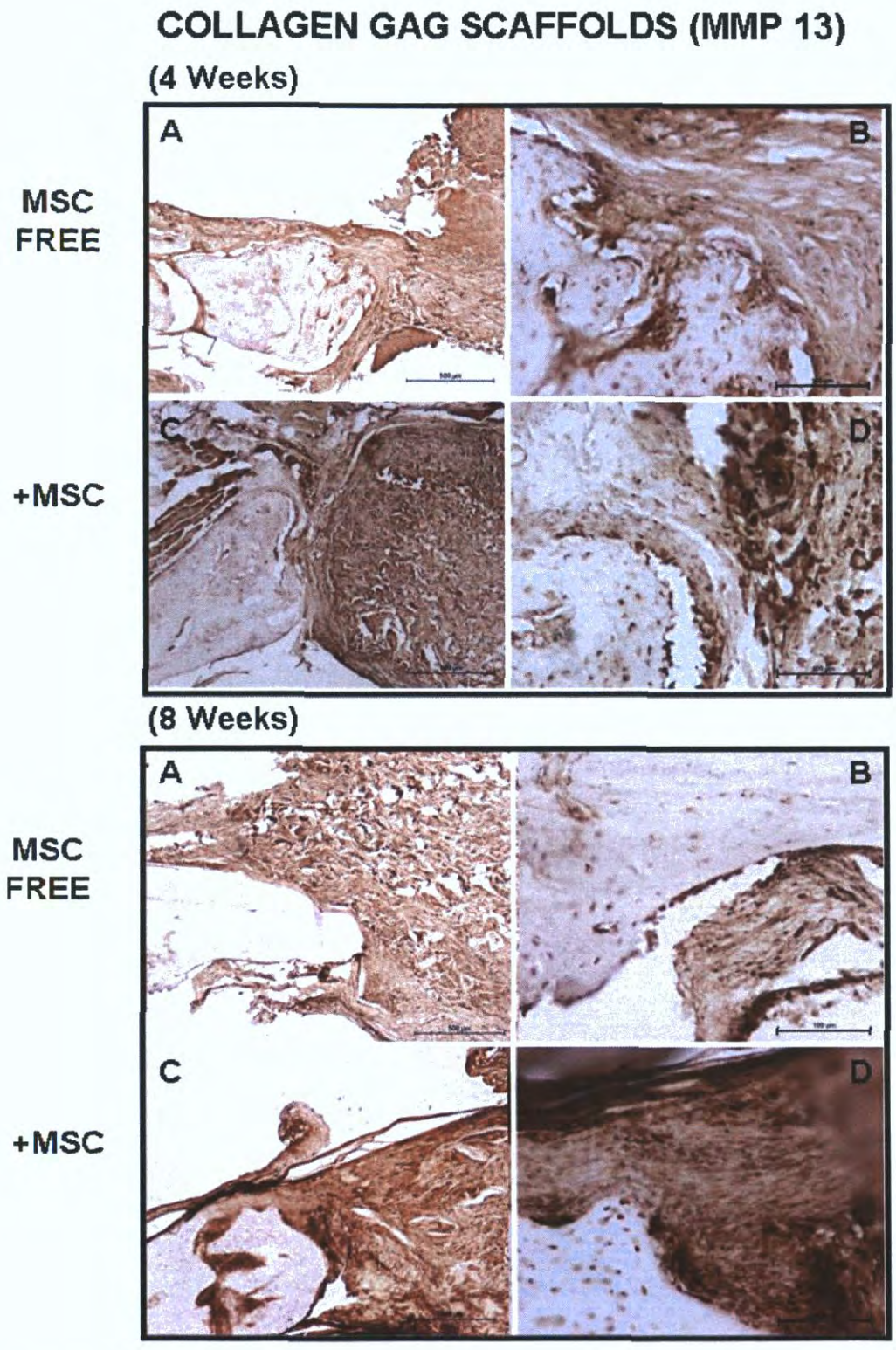

Figure 3.23 *MMP13 immunostaining of collagen GAG scaffolds at both low (x4) and high power (x20) at 4 and 8 weeks.*

CHAPTER 4 – DISCUSSSION

4.1 IN VITRO CHARACTERISTICS OF MINERALISED COLLAGEN SCAFFOLDS

Bone tissue engineering has had limited clinical success to date, in part due to the fact that, an optimal scaffold for engineering bone remains to be established. In order for a scaffold to be successful in bone tissue engineering, a trade-off between sufficient mechanical properties and a porosity and permeability high enough to allow cell migration, tissue formation and angiogenesis is required (Frenkel and Di Cesare 2004; O'Brien, Harley et al. 2007). Our laboratory has developed a novel collagen calcium phosphate composite scaffold which aims to overcome these limitations.

These scaffolds manufactured using a bi-phasic immersion technique have previously been shown to have markedly superior mechanical properties over pure collagen scaffolds whilst maintaining a high rate of porosity (approximately 92%) which is critical for new bone formation (Al-Munajjed and O'Brien 2009). However, modern tissue engineered scaffolds are designed to degrade over time being replaced by new host cells and ultimately new tissue. Such controlled degradation ideally should not exceed the rate of new bone formation and the reduction of strength of the implant should closely match the increase in new tissue strength, otherwise the stresses could be transferred to the healing bone, which could be detrimental for bone healing (Liao and Cui 2004). The aim of the *in-vitro* component of this study was to measure both the degradation characteristics and change in mechanical

properties as these novel scaffolds degraded over time, as compared to pure collagen and collagen GAG controls.

Scaffold degradation characterisation was performed in two separate degradation media, PBS and collagenase in PBS. The PBS only media was used to assess scaffold degradation by hydrolysis alone whilst the collagenase degradation media aimed to partially mimic the *in vivo* conditions of biodegradation at body temperature. *In vivo* collagenases are proposed to be released by osteoclasts and are selectively capable of breaking peptide bonds on each *α* chain of the tropocollagen matrix (Harrington 1996). The use of bacterial collagenase acts to mimic this *in vivo* degradation and also serves to accelerate degradation for experimental purposes.

This study shows that in a degradation media consisting only of PBS, both collagen and Coll-GAG scaffolds do not degrade over a 42 day incubation period. However the novel CCP scaffolds, whilst significantly heavier throughout, degrade by approximately 30% during the first 24 hrs of incubation. This initial degradation ceases with no further degradation over the remaining experimental period. Under conditions of enzymatic degradation with bacterial collagenase both collagen and Coll-GAG scaffolds showed initially slow rates of degradation prior to rapid and complete degradation after a critical time point (12 and 24 hours respectively). In a similar manner to that found in the PBS only media, the CCP scaffolds showed an initial period of degradation, which subsequently plateaued. The CCP scaffolds were resistant to further degradation up to a final time point of

72 hours incubation. It is likely that the initial loss of weight in both degradation media of CCP scaffolds is caused by calcium phosphate particles which have not bound to collagen during the manufacture process re-entering solution. The most important finding of this experiment is that mineralisation of pure collagen scaffolds using a bi-phasic immersion process results in scaffolds that are relatively resistant to complete degradation, when compared to dual cross linked (DHT and EDAC) collagen and Coll-GAG scaffolds. Similar resistance to degradation in scaffolds containing calcium phosphate has been shown by other authors in synthetic polymer based composite scaffolds but not as yet for collagen based composite scaffolds (Baji, Wong et al. 2007; Guarino, Taddei et al. 2009).

It is desirable that tissue engineered scaffolds maintain their mechanical properties after host implantation in order to support the surrounding tissues during wound healing. Furthermore, scaffolds ought to interact with their mechanical surroundings to effectively transmit mechanical stimuli to cells (Guilak 2002) as mechanical stimuli have the ability to modify cell behaviour including cell differentiation (Prendergast, Huiskes et al. 1997). This experiment has shown that collagen, Coll-GAG and CCP scaffolds maintain their compressive strength when immersed in PBS at body temperature for 42 days. However, despite their apparent inhibition to enzymatic degradation in collagenase CCP scaffolds progressively lose their mechanical properties over 72 hours of incubation in collagenase. This finding suggests that CCP are not completely resistant to collagenase degradation and that collagen

fibres are cleaved, resulting in a diminution of mechanical properties prior to complete degradation of the construct.

4.2 IN VIVO NEW BONE FORMATION AND DEGRADATION OF MINERALISED COLLAGEN SCAFFOLDS

The initial In-vitro component of this thesis has demonstrated our novel CCP scaffolds to have improved mechanical properties and a relative resistance to degradation as compared to collagen only and ColIGAG scaffolds. We therefore went on to investigate the ability of the CCP scaffolds to heal a critically sized defect using a rat calvarial model. These results confirm that a 7mm defect is indeed of a "critical size" in a rat calvarial model, with minimal new bone formation at 8 weeks (Mean ± SD; 5.10 ± 2.82%), the defect being predominantly filled with fibrous tissue.

While there are numerous animal models used in the literature, we chose a rat calvarial defect model due to its wide acceptance and well understood physiology and genetic homogeneity (Petrie Aronin, Sadik et al. 2009). Cranial defects seem appropriate for *in vivo* tests, as they do not need further internal or external fixation of the defects, which might introduce extra variability and influence the outcome of the experiment. The scaffold is easily secured in the defect space by over-suturing the periosteum and skin. Furthermore in the calvarium intramembranous ossification occurs (Junqueira, Carneiro et al. 1995). Intramembranous ossification also occurs in the maxilla, mandible and temporal bones; regions of the skeleton which often require

reconstruction following trauma or head and neck oncologic surgery and thus remain the long term clinical direction of this body of work.

NON MSC SEEDED SCAFFOLDS

Both the non-MSC seeded CCP and CollGAG scaffolds showed healing relative to the empty defect controls, the results conclusively demonstrated that the presence of the calcium phosphate phase in the scaffold enhances healing and new bone formation using quantitative histomorphometric assessment. In the non-MSC seeded scaffolds the quantitative data and qualitative images show similar results, with superior healing of the defect and new bone formation using the CCP scaffolds. Some specimens showed almost full healing of the defect after 8 weeks compared to a slower rate of healing in the CollGAG scaffolds.

The CCP scaffolds superior results are likely due to two factors: At a cellular level, it is recognised that the presence of any form of calcium-phosphate, especially hydroxyapatite acts as an osteogenic factor *in vitro* and *in vivo (Denissen, de Groot et al. 1980; Hammerle, Olah et al. 1997)*. Calcium phosphate coatings are widely used in many orthopaedic (Kikuchi, Itoh et al. 2001) and dental (Yap, Pek et al. 2002) implant materials as it is bioactive and it improves osteoconductive and osteoinductive properties. In addition, in bone tissue engineering, CP coatings are used to adhere mineral phases onto synthetic and biological polymer scaffolds to increase their osteoconductive and osteoinductive performance (Muller, Muller et al. 2006). The second factor that explains the superior results for the non MSC seeded CCP scaffolds compared to the CollGAG scaffolds is their improved mechanical

properties. Whilst the rat calvarium is not a weight bearing bone, some forces are applied to the scaffold both intra-operatively and post-operatively particularly in view of the fact that 7mm defect is large relative to the rat skull. Soft tissue wound closure over the defect is likely to have exerted some compressive forces onto the scaffold, which the stronger CCP scaffolds would be more easily able to withstand. These mechanical properties allow the 3D pore structure to maintain its configuration, permitting cell infiltration into the scaffold structure.

MSC SEEDED SCAFFOLDS

Those scaffolds implanted after prior culture with MSCs promoted new bone formation however at a slower rate when compared to non MSC seeded scaffolds for both the CCP and CollGAG groups. Previous work with both of these scaffolds *in vitro* have demonstrated ECM deposition and mineralisation when pre-cultured with MSCs over a 28 day period (Al-Munajjed, Lyons et al. 2010).

Histological assessment demonstrated a dense layer of connective tissue "capsule" around both scaffold types 4 weeks post implantation in the rat calvarium, potentially preventing nutrient flow into the scaffold centre and leading to a cell-free area. This encapsulation effect was even more pronounced in the CCP scaffolds due to a higher level of ECM deposition after the *in vitro* cultivation period. The remodelling of this dense layer slows down new bone formation *in vivo* possibly explaining the difference in the results compared to the non MSC seeded scaffolds. Similar immune host

response to cell seeded scaffold materials leading to slower new tissue formation has been reported using an autologous cellular construct in a rat model (Brown, Valentin et al. 2009) and using ECM based scaffold in a primate model (Xu, Wan et al. 2008). This latter study showed the presence of cellular content was associated with increased levels of pro-inflammatory cytokines, increased macrophage activation, and a poor remodelling outcome.

These scaffolds were cultured with MSCs for 28 days prior to implantation. This is potentially too long a period, as a dense cellular matrix aggregates at the periphery of the scaffold without cellular penetration into the substance of the scaffold. The optimal duration for *in vitro* culture of scaffolds prior to implantation has not been clearly established, with *in vitro* studies examining osteogenesis over different time points.

IN VIVO DEGRADATION

Scaffold degradation has an important and complex relationship with the cellular processes involved in successful new tissue formation in a tissue engineered construct including, cell growth, tissue regeneration and host response (Liao and Cui 2004). Whilst scaffold degradation is traditionally measured using in vitro models with different degradation media (such as PBS and collagenase in the first part of this thesis), such models over simplify the true environment once implanted. In vivo assessment of degradation is however limited by the fact that it is difficult to determine the exact extent of scaffold weight loss, as the scaffold has been partially replaced by new tissue and the remaining scaffold can not be easily separated from the surrounding host tissue. One alternative strategy is to implant the scaffold material into an

animal subcutaneously (Gong, Zhou et al. 2007). However, this approach would fail to accurately mimic the complex cellular, immune and cytokine interactions at sites of bone fracture or new bone formation.
Using histomorphometry we have overcome these limitations, permitting an approximate measurement of in vivo degradation at the intended implant site and in the presence of new tissue formation.

Histomorphometry of the area of remaining scaffold revealed that almost complete degradation of the non MSC seeded CollGAG scaffolds at 8 weeks with a slower rate of degradation in the non MSC seeded CCP scaffolds. These CCP scaffolds did show a significant progression in scaffold degradation from 4 to 8 weeks. These findings corroborate the findings of the in vitro degradation studies that the CCP scaffolds show retarded degradation compared to crosslinked CollGAG scaffolds. For both CCP and CollGAG MSC seeded scaffolds, little if any degradation was seen at either time point. This finding would add support to the previous theory that the MSC seeded scaffolds' dense fibrous capsule hinders both cell infiltration as well as other mediators including collagenolytic enzymes into the centre of the scaffold, thus inhibiting scaffold degradation.

4.3 HOST IMMUNE RESPONSE TO IMPLANTED COLLAGEN SCAFFOLDS

MACROPHAGE PHENOTYPE

In order to further characterise the apparently excessive inflammatory response (resulting in the dense fibrous capsule) to the MSC pre-cultured scaffolds, immunohistochemistry was performed to determine the level and site of macrophage recruitment to the defect site. Macrophages play an important role in the initial response to fracture healing (Schindeler, McDonald et al. 2008) and it seems logical that they would have a similar role in the early stages of the healing process following the construction of the calvarial defect and implantation of the scaffold materials. Furthermore, the ability to phenotype macrophages immunohistochemically as either a pro-inflammatory M1 response associated with the deposition of dense connective tissue and / or scarring, or the M2 response associated with constructive remodelling was utilised (Brown, Valentin et al. 2009).

Allogenic MSCs are thought to be nonimmunogenic or hypoimmunogenic due to their absence of HLA Class II expression and low expression of co-stimulatory molecules and may even actively hinder T-cell production (Krampera, Glennie et al. 2003; Majumdar, Keane-Moore et al. 2003). However, scaffolds themselves can induce variable immune responses, which are generally characterised by an intense macrophage infiltrate, which may be affected by the scaffold materials, manufacturing techniques and presence of cellular tissue (Badylak and Gilbert 2008). This immunohistochemical work aimed to identify whether macrophages played a role in the host response to

the implanted scaffolds and if so, to determine whether the response was of the inflammatory M1 type or tissue remodelling M2 type.

Whilst our data is only qualitative, a clear trend emerged; namely that there was a macrophage response to all scaffolds, which was abundant and constrained to the periphery of MSC seeded scaffolds but weakly present throughout the non MSC scaffolds. This response seemed to dissipate earlier in the non MSC seeded CCP scaffolds compared to the non MSC seeded CollGAG scaffolds. The pro-repair and tissue remodelling M2 phenotype response was evident at sites of new bone formation in both CCP and CollGAG scaffolds, with maximum activity seen at the islands of new bone formed in the core of the CCP scaffolds at 4 weeks and at the host-new bone interface in the CollGAG scaffolds. Non MSC cultured scaffolds demonstrated little M1 phenotype macrophage response. This finding of a marked M2 (tissue remodelling) response at 4 weeks in the non MSC seeded CCP scaffolds which have been shown to result in maximal new bone formation, suggests that an early and pronounced M2 response is important for tissue and scaffold remodelling including new bone formation.

Staining for both M1 and M2 phenotype macrophages in both types of scaffolds which were MSC pre-seeded was positive for both markers but again isolated to the dense fibrous tissue at the periphery of the scaffold. This finding suggests that whilst an attempt at remodelling (M2) is occurring, these macrophages are denied access to the substance of the scaffold by the dense

fibrous tissue that is associated with pro-inflammatory (M1) macrophage activity. These findings are summarised in **Figure 4.1**.

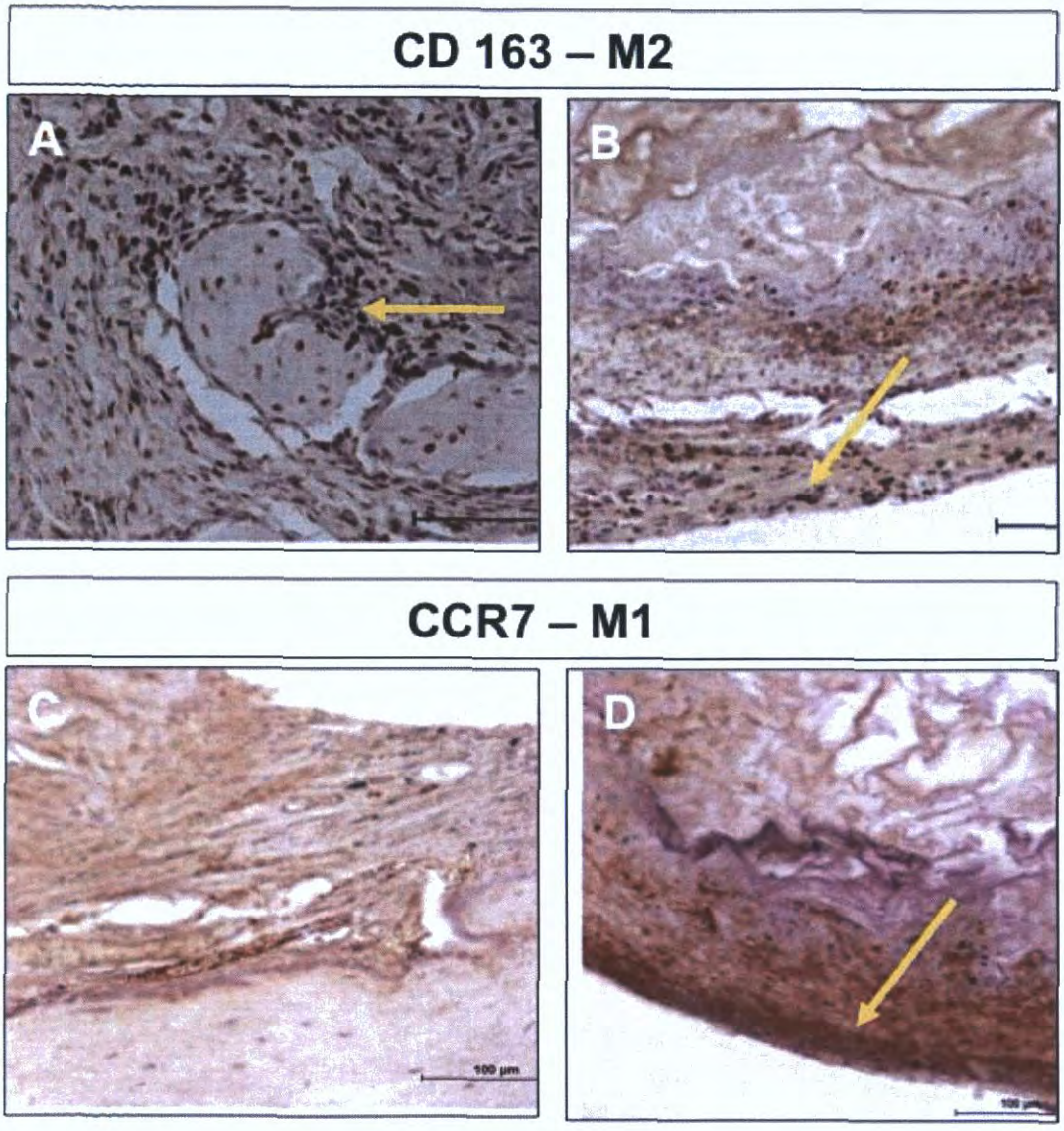

Figure 4.1 *Summary of macrophage phenotype findings. M2 (wound remodelling) macrophages were seen at sites of new bone formation in non MSC seeded CCP scaffolds (A) but at the periphery of MSC pre-seeded CCP scaffolds (B). M1 phenotype macrophage activity is weak / absent in non MSC seeded CCP scaffolds but strongly positive in dense connective tissue capsule seen surrounding the MSC pre-seeded CCP scaffolds (Positive staining indicated by yellow arrows)..*

This finding that the incorporation of a cellular component into a scaffold construct alters the macrophage response from M2 towards the M1 phenotype is consistent with the findings of Brown et al. These authors found that the incorporation of a cellular component onto an extracellular matrix scaffold elicited a predominantly M1 type response, when implanted into the abdominal wall of rats, and resulted in deposition of dense connective tissue (Brown, Valentin et al. 2009). Badylak et al. have shown that carbodiimide crosslinked small intestinal submucosa derived scaffolds (SIS) elicited a M1 phenotype response as opposed to a predominantly M2 phenotype response in non crosslinked SIS scaffolds (Badylak, Valentin et al. 2008). This work suggests that the macrophage response to collagen based scaffolds is not altered by either DHT or EDAC crosslinking but is more dependent upon the presence or absence of a cellular component.

The fact that stem cell seeded scaffolds exhibit a predominantly pro-inflammatory response is not necessarily due to the presence of the stem cells themselves. Rather, the fact that these scaffolds have been cultured in osteogenic media, containing; dexamethasone, β-glycerophosphate and ascorbic acid-2-phosphate, for 28 days. This chemical combination appears to play a role in the deposition of a dense ECM matrix around scaffolds. The presence of such a matrix may play a role in driving the recruited macrophages towards a M1 phenotype rather than a pro-tissue remodelling M2 phenotype. To assess this hypothesis, future work could be performed on scaffolds implanted immediately following MSC application without any exposure to an osteogenic culture media.

COLLAGENASE 3 / MMP-13

The collagenolytic enzyme MMP-13 is known to play a role in fracture repair and bone remodelling (Kosaki, Takaishi et al. 2007). Whilst intense staining was evident at sites of intense new bone formation, few other differences existed between the staining for this enzyme between the empty defect, CCP and CollGAG specimens or between the MSC pre seeded and non MSC seeded scaffolds. Analysis was hampered by diffuse background staining of the collagen scaffolds themselves. Only limited conclusions can be drawn, other than to say that MMP-13 plays a role in the host response to a surgically created calvarial defect.

4.4 FUTURE WORK

This work has demonstrated how the addition of a mineral calcium phosphate component to a collagen scaffold retards scaffold degradation. Ideally it should be possible to tailor the rate of degradation to meet the expected rate of new tissue formation. The freeze drying techniques utilised to manufacture these collagen scaffolds can be altered to adjust pore size (O'Brien, Harley et al. 2004), resulting in a controllable alteration in degradation characteristics. Furthermore, the effect of saliva on the rate of degradation of these has not been assessed to date, if these scaffolds are to be used in reconstruction of mandibular defects, intra-operative exposure to saliva seems inevitable. Furthermore, if such scaffolds were to be used in a dental or orthodontic role continuous exposure to saliva is expected.

Saliva is a mixture of secretions produced by the salivary glands (parotid, submandibular and submental) and the gingival crevicular fluid (a transudate of plasma through the sulcular epithelium). The various functions of saliva are indicated by its different constituents. Mucins serve to lubricate food and keep the mouth moist. Alpha – amylase commences starch digestion, whilst immunoglulin A and lysozyme are part of the immune defence system. Saliva has a low NaCl concentration and is hypotonic. Whilst not typically thought of as a proteolytic secretion, metalloproteinases, including collagenases, have recently been shown to exist in saliva (probably derived from the gingival crevicular fluid) (Chaussain-Miller, Fioretti et al. 2006). Therefore, the effect of saliva on the degradation rate of these scaffolds is an important variable if they are to be used in the oral cavity and mandible region.

The host immune response to implanted tissue engineered constructs has received only limited scientific attention. This work supports the findings of Brown et al that constructs which contain a cellular component modulates the phenotype of the macrophages participating in the host response. One limitation of this work is that scaffolds were cultured with MSCs for 28 days, this may be too long a period that contributes to the excessive inflammatory response seen. Future work, should examine shorter culture times including MSC application to the scaffold immediately prior to implantation. Future work into the host immune response to biologic scaffold materials can only lead to greater safety and efficiency of the resultant devices.

The ultimate goal of this body of work is to develop tissue engineered alternatives to bone grafting and bone tissue transfer. Whilst showing encouragingly superior mechanical properties over CollGAG scaffolds this study tested the CCP scaffolds in a non load bearing anatomical location. Future work is planned to incorporate such mineralised collagen scaffolds in weight bearing long bones. The oral cavity and mandible have a number of cellular, mechanical and environmental differences to other skeletal regions, for these scaffolds to be successfully and routinely incorporated into human head and neck region defects, further work needs to be performed to understand and optimise their performance in this unique and challenging anatomical location. To realise this goal, the next logical progression would be to utilise these scaffolds in a mandibular model. A suitable validated critically sized mandible defect model exists and would be appropriate to test these

scaffolds in a larger defect, whilst exposed to increased mechanical forces and the physiologic microenvironment of the oral cavity.

4.5 CONCLUSIONS

- The addition of calcium phosphate to highly porous collagen scaffolds results in a decrease in the in vitro rate of degradation of these scaffolds, which is predominantly medicated by enzymatic collagenolytic digestion. Furthermore, these mechanically stiffer scaffolds lose their mechanical properties in a progressive fashion in the collagenolytic degradation media. In vivo assessment of degradation using histomorphometry confirmed retarded degradation in the mineralised calcium phosphate scaffolds.

- Composite collagen Calcium Phosphate scaffolds (without prior culture in mesenchymal stem cells) exhibit significantly superior rates of new bone formation than collagen-GAG controls at both 4 and 8 weeks implantation into a rat calvarial model.

- Prior culture of culture of Composite collagen Phosphate scaffolds and collagen GAG scaffolds with mesenchymal stem cell cells for 28 days results in lower rates of new bone formation than their non cell seeded counterparts. However, new bone formation was again higher in the collagen Calcium Phosphate scaffolds that were stem cell seeded than the collagen GAG scaffolds that were stem cell seeded.

- Mesenchymal stem cell seeded scaffolds are characterised by a dense fibrous and inflammatory capsule which hindered new bone formation and scaffold degradation. This inflammatory reaction is macrophage mediated via a pro-inflammatory M1 phenotype macrophage response. Whilst superior new bone formation in non cell seeded scaffolds is associated with an M2 tissue remodelling macrophage response.

APPENDIX

APPENDIX I Freeze Dryer Recipe 5

Thermal Treatment

	Temperature (°C)	Time (mins)	Process
1	+ 20	20	Hold
2	- 40	60	Ramp
3	- 40	60	Hold

Freeze Condenser, vacuum phases

Freeze (°C) = - 40
Additional Time (mins) = 00
Condenser (°C) = - 55
Vacuum (mT) = 200

Drying Cycle

	Temperature (°C)	Time (mins)	Process	Vacuum
1	- 40	05	Hold	200
2	0	250	Ramp	200
3	0	1020	Hold	200
4	20	20	Ramp	200

Secondary Drying

Setpoint = 65
Post Heat Setting
Temperature (°C) = + 20
Time (mins) = 70
Vacuum (mT) = 200

APPENDIX II Haematoxylin & Eosin (H&E) Protocol

Procedure		
Deparaffinise	Xylene	5min
Hydrate	100% Ethanol x2	30 sec
	90% Ethanol	30 sec
	70% Ethanol	30 sec
Rinse in Water		
Stain	Harris Haematoxylin	15min
Rinse in Water		
Differentiate	1% Acid Alcohol	20-30 sec
Rinse	Tap water	"Until Blue"
Stain	0.5% Eosin	1-2 min
Rinse in Water		Till clear
Dehydrate	70% Ethanol	30 sec
	90% Ethanol	30 sec
	100% Ethanol	30 sec
Clear	Xylene x 2	1 min
Mount & Coverslip	DPX	

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